VOLUME 8 July 1959 NUMBER 4

THE AMERICAN JOURNAL OF

Tropical Medicine and Hygiene

Combining The American Journal of Tropical Medicine and The Journal of the National Malaria Society

OFFICIAL ORGAN OF
THE AMERICAN SOCIETY OF TROPICAL MEDICINE AND HYGIENE

PUBLISHED BIMONTHLY BY
THE WILLIAMS & WILKINS COMPANY
BALTIMORE 2, MARYLAND, U.S.A.

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THE AMERICAN JOURNAL OF

Tropical Medicine and Hygiene

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THE AMERICAN JOURNAL OF TROPICAL MEDICINE AND HYGIENE is issued bimonthly, appearing in January, March, May, July, September, and November. One volume is issued per year.

Correspondence concerning business matters, subscriptions and orders for reprints, should be addressed to The Williams & Wilkins Company, 428 East Preston Street, Baltimore 2, Maryland, U. S. A.

Manuscripts and Books for Review should be sent to Dr. Martin Frobisher, 374 Ruckman Road, Closter, New Jersey.

New subscriptions and renewals are entered to begin with the first issue of the current volume. Should any issue of the current volume be out of print at the time the subscription order is received, the pro rata value of such numbers will be refunded to the subscriber.

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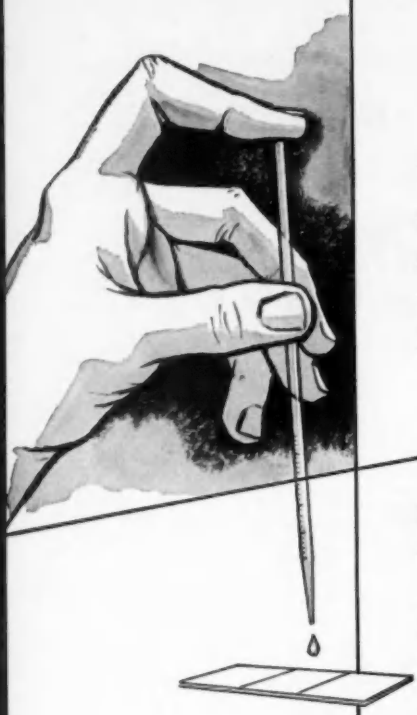

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Subscription information: Published monthly. Subscription price per year: $15.00. Foreign postage: $1.50. Canada postage $.50. Volumes 82-83 current in 1959.

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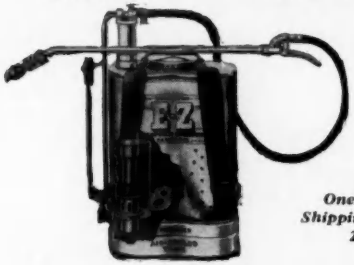

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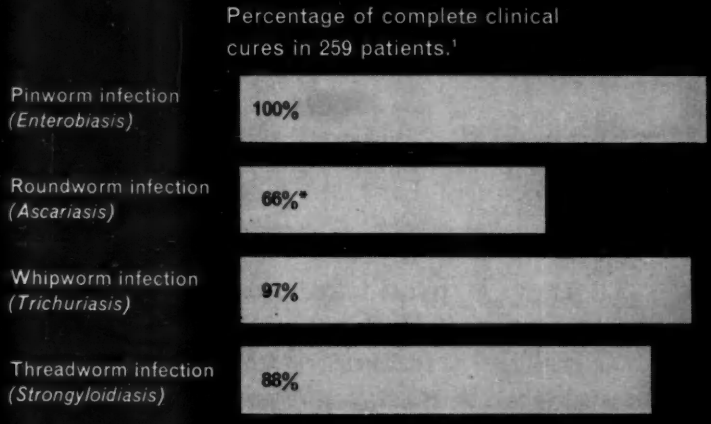

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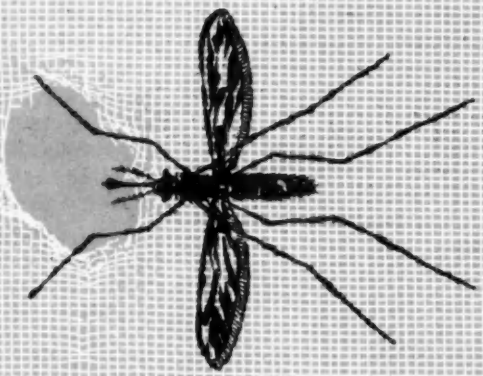

THE EFFECTS OF CHEMICAL AND PHYSICAL AGENTS ON FILARIFORM LARVAE OF *ANCYLOSTOMA BRAZILIENSE**

HARVEY BLANK, MARY W. WINTER AND J. WALTER BECK

Division of Dermatology of the Department of Medicine and the Department of Microbiology, University of Miami School of Medicine, Miami, Florida

An effective treatment for the distressing skin lesions of creeping eruption caused by the filariform larvae of *Ancylostoma braziliense* is urgently needed. Experienced clinicians have found that the most reliable remedy is freezing the skin for an inch in all directions around the advancing edge of the tract with ethyl chloride, Freon or solid carbon dioxide for 30 to 60 seconds. Experiments in our laboratory (unpublished) indicate that this procedure, introduced by Shelmire[16] in 1905, does not kill the larva but apparently causes its mechanical desquamation in the blister tops and crusts induced by the burn-like trauma of prolonged freezing. In many patients this painful procedure is impractical or impossible and effective chemotherapy is indicated. In severe cases some partial temporary symptomatic relief can be obtained by systemic administration of adrenal glucocorticoids of which triamcinolone seems best, but it cannot compare with what one would hope to achieve with an active larvicidal drug. There is also a persistent need for more effective agents to kill the larvae and ova in gardens, athletic fields, dog runs and similar contaminated soils. The present study is a report of our attempts to find such compounds.

A number of agents have been suggested for the chemotherapy of creeping eruption. Many of the reports are based on the observation of clearing of skin lesions following the systemic administration of a particular drug. Various forms of antimony, tartar emetic, stibophen, ethylstibamine, etc., as well as oxophenarsine HCl, were thought to be effective by some authors,[6, 13, 17, 21, 22] but were reported as without value by others.[3-5, 14] Diethylcarbamazine was described as useful by some workers,[8, 9, 20] but judged as disappointing by others.[11, 15] The probable reason for these discrepancies in clinical evaluations was brilliantly portrayed by Loewenthal[10] in his study of the natural course of the infection in man. Creeping eruption is a self-limiting disease in which the vast majority of lesions clear spontaneously in several weeks to several months, although an occasional larva may persist for a year. As a result, the current treatment is apt to be given credit for a spontaneous cure. Sompayrac[18] and Beaver[2] also warned against this danger. Before ascribing clinical efficacy to any form of systemic therapy, the results must be compared, as described by Loewenthal,[10] with similar untreated lesions.

The difficulties inherent in the clinical evaluation of therapeutic agents for creeping eruption have encouraged previous workers, as well as our own group, to test agents *in vitro* against the third stage filariform larvae of the dog and cat hookworms. Several investigators[11, 12] found that the heavy metals and diethylcarbamazine, along with antibiotics and a number of other compounds, were essentially ineffective even at concentrations (1 to 5 per cent) far exceeding those attainable in a patient. Full strength solvents such as alcohol or acetone were lethal. The only drug which they found active when diluted was hexylresorcinol, 0.06 per cent aqueous. Of 12 other resorcinols only the butyl, octyl and amyl showed some inactivating action. They concluded that the larvae are not easily killed by drugs which might be used therapeutically. Studies carried out by McCauley (in Donaldson, *et al.*[5]) and by Hoerlein,[7] concluded that for sterilization of the soil, calcium cyanamide, methyl bromide, sodium borate and urea showed varying degrees of effectiveness.

MATERIALS AND METHODS

All studies were undertaken with pure cultures of *Ancylostoma braziliense*. Approximately 80 female and 30 male adults of this species were selected from the small intestines of autopsied dogs and fed in gelatin capsules to puppies in whom previous infection had been excluded by repeated negative stool examinations. On the

* This study was supported by a Research Grant from the Public Health Service (E-1228-C2).

second day after the capsules had been fed to the puppies, ova were found in the stools of the infected animals. Subsequently other clean puppies were successfully infected by subcutaneous inoculation of approximately 1,000 filariform larvae obtained from culture of the stools of the first animals. Infections persisted with either method providing infected feces for study at all times.

Copraculture with sterile sand (to exclude free living nematodes) and subsequent separation of the larvae with a Baermann apparatus, after 6 days' incubation at room temperature, resulted in organisms which required further decontamination. By allowing the larvae to gravitate through a column of sterilized sand attached to the Baermann apparatus, as Avery[1] described for separating trichinae larvae from bacteria, relatively clean organisms were obtained. Suspensions of the sheathed larvae were stored at room temperature until used. In most experiments the organisms were used promptly, but storage for several weeks seemed to have little deleterious effect on them.

Many of the drugs to be tested were insoluble in water and the following technic was devised for such compounds. They were first dissolved in N,N-dimethylacetamide containing 10 per cent of Triton X-100. This is almost a universal solvent for organic compounds. Subsequent dilutions were made with the solvent so that at the 6 dilutions in water which were always tested, the same amount of solvent (5 per cent) was present and only the amount of active drug varied. A solvent as well as water control was also run. If water soluble, this solvent was omitted. The final concentrations of the drugs were always 10, 50, 100, 500, 1,000 and 5,000 μg per ml. Approximately 30 larvae were pipetted into deep well slides and the excess water removed under binocular microscope control. To each well 0.3 ml. of diluted drug was added and the wells were then covered to prevent evaporation. The larvae were observed at hourly intervals and the next day at 24 hours. A note was made of failing larvae but the least difficult end point proved to be death, or failure of movement, of all larvae. All determinations were repeated, many important ones were done four or five times with intervals of months between tests.

TABLE 1

Activity against A. braziliense *in vitro*

Antibiotics*	Solvents*
Acti-dione (cycloheximide)	ethyl alcohol
Fungizone (amphotericin B)	polyethylene glycols
Griseofulvin	propylene glycol
Mycostatin (nystatin)	N,N-dimethylacetamide
Achromycin (tetracycline)	N,N-dimethylformamide
Aureomycin (chlortetracycline)	Triton X-100
Cathomycin (novobiocin)	
Chloromycetin (chloramphenicol)	
Penicillin	
Streptomycin	
Terramycin (oxytetracycline)	

* All were ineffective at 0.5% or less.

RESULTS

A total of 183 drugs was tested. The results of the more active ones are given in some detail in the tables. The column headings indicate the concentration of the drug and the numbers in each column indicate the number of hours in which all larvae were inactive, except that 0 indicates no lethal effect. In Table 1, the inactivity of commonly used antibiotics and solvents, including those used in this study, is indicated. In Table 2, many of the active common antiparasiticides are listed. At the top of this list, but of only moderate activity, is one of the newer soil nematocides, Vapam® (sodium N-methyl dithiocarbamate, Stauffer Chemical Co.). Other soil nematocides, however, including D D (dichloropropenedichloropropane, Shell Chemical Co.) and Nemagon® (1,2-dibromo-3-chloropropene, Shell Chemical Co.) and VC-13 (O-2,4-dichlorophenyl - O,O - diethylphosphothioate), were of surprisingly little value under conditions of this experiment.

Among the various phenothiazene derivatives, the most active *in vitro* was Thorazine® (chlorpromazine, Smith, Kline and French).

The chlorinated hydrocarbon insecticides were studied not only because there were no previous reports of their activity, but also because many of them penetrate intact skin and presumably could get to the larvae following topical application. Topical lindane (gamma isomer of benzene

TABLE 2

*Active Compounds**

	Antimalarials, antihelmin-thics, antiseptics					
	10	50	100	500	1,000	5,000
Vapam	0†	0	0	8	6	4
Abbott 6839	0	0	24	12	8	4
Abbott 502	0	0	0	24	19	insol.
Merthiolate	0	0	0	0	0	24
Primaquine	0	0	0	0	0	24
Chlorinated hydrocarbons						
Bulan	0	0	0	24	12	t.‡
Dilan	0	0	0	24	t.	t.
Dieldrin	0	0	0	0	24	24
Prolan	0	0	0	0	24	t.
Lindane	0	0	0	0	24	12
Aldrin	0	0	0	0	0	2
D D	0	0	0	0	0	1
Nemagon	0	0	0	0	0	24
Phenothiazines						
Chlorpromazine HCl	0	0	24	8	6	6
Promazine HCl	0	0	0	10	10	10
Trimeprazine	0	0	0	0	24	24
Promethazine HCl	0	0	0	0	24	24
Trifluprom azine HCl	0	0	0	0	0	8
Organic phosphates						
Co-ral	0	24	24	t.	t.	t.
Malathion	0	0	0	12	12	t.
Diazinon	0	0	0	24	12	t.
Ethyl *p* - nitrophenyl-benzinephosphonate	0	0	0	24	24	t.
Systox	0	0	0	24	24	12
Vapotone	0	0	0	24	12	8
VC 13	0	0	0	0	24	t.

* Values given as μg/ml.

† Zero indicates no lethal effect.

‡ t., turbid.

hexachloride) is used as a 1 per cent cream for the treatment of scabies in man but was not very active *in vitro* against the filariform larvae. Dilan (1 part Prolan and 2 parts Bulan) is much less toxic than dieldrin, probably because it is poorly absorbed[19] although they are of equal activity *in vitro*.

The organic phosphates were tested because they, too, are not only active against many parasites but also are readily absorbed through intact skin. The most toxic, such as tetraethyl pyrophosphate and parathion, were not used. All members of this group, with the exception of one, had *in vitro* activity of the same range. The outstanding compound was Co-ral (O,O-diethyl O-(3-chloro-4-methyl-7-coumarinyl) phosphorothioate, Chemagro Corp.; formerly Bayer 21/199). It is said to be of low toxicity and is presently used as a drench on the skin of cattle to control screw worm, cattle grubs, lice, ticks, etc. Following topical application it is thought to be absorbed and to exert a systemic effect.

TABLE 3

*Inactive compounds**

Antimalarials, antihelmin-thics, antiseptics, etc.	Chlorinated hydrocarbons
Hydroxychloroquine SO_4	Chlordane
Quinacrine HCl	DDT
Chloroquine PO_4	Endrin
Piperazine citrate	Heptachlor
Arsthinol	Methoxychlor
Dithiazanine iodide	Toxaphene
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Polyborates	
o-Phenylphenol	O-(2-trimethylammo-nium) propyl phenothi-azine methyl sulfate
Benzalkonium chloride	10-Dimethylaminopropyl-phenothiazine HCl
	Prochloroperazine ethanedisulfonate

* An additional 122 compounds of many types which were submitted by chemical and pharmaceutical manufacturers also were found inactive.

In addition to the compounds listed in Table 2, many others were also tested but showed no activity, or of such a low degree as judged to be of no value (Table 3); these included 94 compounds submitted by Abbott Laboratories, 11 submitted by Searle and 18 by Merck, and designated only by code numbers.

SUMMARY

A study in vitro was performed, testing 183 compounds against the filariform larvae of *Ancylostoma braziliense*.

As previous workers had found, the larvae are resistant to many commonly used parasiticides.

Among the organic phosphates, Co-ral (O,O-diethyl O-(3-chloro-4-methyl-7-coumarinyl) phosphorothioate) and malathion had activity which might be of practical value in the treatment of human infections and for application to contaminated soil. Dilan among the chlorinated hydrocarbons, chlorpromazine among the phenothiazines and Vapam® among the nematocides showed similar activity.

The importance of spontaneous cure of creeping eruption in the evaluation of drug therapy is discussed.

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PERSISTENCE OF BACTERIA IN THE DEVELOPMENTAL STAGES OF THE HOUSEFLY

I. Survival of Enteric Pathogens in the Normal and Aseptically Reared Host*

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The tiled lavatories and sanitary drains of Ur and Kish dating from 3,000 B.C., and Moses' edict to the Israelites to bury their excreta, suggest that a few early civilizations had already come to grips with environmental health problems through sanitation. Some relationship between the housefly and public health was probably appreciated by the ancient Egyptians who understood the complete life cycle of the fly. Unfortunately, even this level of appreciation was lost during the Middle Ages when maggots and other "lowly" creatures were thought to arise spontaneously from excrement and waste. But gradually with time, facts of nature derived from direct observation began to accumulate. In the 17th century, the life cycles and structure of insects began to be studied with zeal, but it was not until the start of the 20th century, with the development of bacteriology, that serious studies were undertaken to determine the role of the fly in the spread of disease. So great was the interest in this field, that more work was published during the first two decades than has since appeared.

One problem which drew many investigators dealt with the fate of pathogenic bacteria ingested by maggots in the breeding media. Successful passage of such organisms from larva to adult would enhance the vector potential of the fly by aiding in the survival and dissemination of these organisms. This approach was vigorously pursued by many pioneer workers, among whom Faichie[1] was one of the earliest to successfully pass typhoid and paratyphoid organisms in this way. His results are questionable, however, because of possible recontamination of flies emerging from unsterilized puparia. Ledingham[6] was unable to recover *Salmonella typhi* from pupae until recourse to aseptic rearing. He was then able to isolate the pathogen from larvae and from one pupa. Graham-Smith[3] used sterilized media for the larvae and sterile food for the adults, but did not sterilize the eggs nor prevent aerial contamination of the larval medium. Under these circumstances, neither *S. typhi* nor *Salmonella enteritidis* was ever isolated from the adult. *Proteus morgani* was persistently present, which typified the species of nonlactose fermenters which are adapted to the larval intestine and can withstand metamorphosis, according to Graham-Smith. With complete asepsis, Tebbutt[10] recovered *Shigella dysenteriae* from pupae and adults in a small number of cases, but not *S. typhi*. He recovered fewer *S. dysenteriae* than 'Bacillus A', possibly an unidentified species of *Proteus*. The early consensus was that species of *Salmonella* and *Shigella* did not typically survive pupation in the housefly and such other Muscoidea as *Calliphora* sp., *Sarcophaga* sp., and *Sarcophagula* sp.[3, 5, 6, 8, 10, 11] Augmenting the difficulties attending these pioneer efforts was the unavailability of highly selective media for the routine isolation of enteric pathogens. More recently, Ostrolenk and Welch[9] demonstrated the presence of *S. enteritidis* in the emerging adult housefly. Since pools of larvae or adults were tested, we do not know what percentage of emerging adults was infected.* Gerberich[2] found about 41 per cent of emerging flies infected with *Salmonella pullorum*.

The phases which we have investigated, and will report in this and subsequent papers, may be defined as follows:

1. A comparison of the survival of *Salmonella* and *Shigella* species in fly stages breeding in a

* This study was supported by a grant from the University of Illinois Research Board.

The author gratefully acknowledges the helpful interest of Dr. Howard J. Shaughnessy, Professor of Public Health, University of Illinois, College of Medicine, and the contribution of Miss Esther Cooksey of Chicago in the preparation of the charts.

* The term infection is used throughout to mean presence of bacteria within the host whether or not multiplication, tissue invasion or mechanisms of host resistance are demonstrable.

normally contaminated larval medium with those breeding aseptically.

2. Quantitative study of the host-contaminant relationship in flies breeding under natural conditions.

3. Quantitative study of the distribution of bacteria in the host.

The above categories embrace an effort to obtain a continuous picture of the quantitative distribution of bacteria harbored by the fly, from early maggot to young adult. When quantitative methods have been applied to housefly transmission studies, the focus has generally been on the adult. The present study is the first quantitative effort we are aware of which attempts to fill in the preadult gap with information about the vector-contaminant association so essential to an understanding of the life of the fly, not alone as a disease vector, but as an organism which invites exploration for its own sake.

MATERIALS AND METHODS

Transmission Procedure

Techniques for routine and axenic culture of houseflies (*Musca domestica* L.) in CSMA* have been described.[4] Maggots, growing in a normally contaminated medium, were infected with the test species of bacteria as follows. The larval medium consisted of 14 gm of CSMA moistened with 30 ml of tap water, or multiples thereof, in Erlenmeyer flasks. This was seeded with untreated eggs taken from the feeding dishes in the fly cages and kept at 36°C. When maggots reached the 3rd instar, 5 ml of a 24-hour broth culture of the test organism were pipetted into the medium. The species studied were *Shigella flexneri* IIa, *S. typhi* (Vi) and *Salmonella paratyphi* B.† Unplugged flasks were then placed in a tray containing $HgCl_2$ solution, in which migrant larvae were drowned and disinfected. To test the maggots' infectivity, 8 to 10 specimens were removed 1 day after introducing the pathogen, disinfected and individually ground. The homogenate was pipetted into tetrathionate broth which was incubated for 18 hours at 37°C. Isolations of the test organism were made on bismuth sulfite agar and/or SS agar, depending on the species. For diagnostic and confirmatory tests, we used Kligler's iron agar and slide agglutination with appropriate antiserum. Two-day-old pupae, taken from the larval medium, were disinfected and kept singly at room temperature in corked tubes containing moist, sterile sand. Adults, less than 30-minutes old, were processed like the maggots, but were not washed or disinfected.

For axenic rearing, 200 to 300 disinfected eggs were pipetted into the sterilized larval medium, and a smaller number into thioglycollate broth for a sterility test. An additional sterility test of the larval medium was made when the broth culture was added. This was added at the same time and in the same volume as before. We also used *S. enteritidis* in this study. The migratory urge of the prepupa caused it to burrow through the cotton plug and introduce contamination. If wood shavings or vermiculite‡ were added to early 3rd instars, pupation occurred in these materials. At 35°C, the pupa stage lasts 4 days. After the first day, the pupa has molted; a transparent molting membrane envelops it and its head is usually uneverted. At 2 days, the head is everted and the compound eyes are amber. Eye color, at 3 days, is orange or brown and bristles are slightly pigmented. Eclosion occurs during the next day or so. Mature 3rd instar maggots were disinfected and each separately ground. Pupae of specified age were removed from the disinfected puparium and ground without washing or disinfection. Appropriate dilutions of these homogenates were mixed with BHI§ agar as pour plates. Plates were incubated 2 to 3 days at 36°C and the final count for a specimen was the average of 2 replicates. To detect *Proteus* and other contaminants whose colonies are similar, 4 colonies from each specimen were inoculated into Kligler's iron agar and confirmed by slide agglutination using appropriate antiserum. When contamination was present, the plates were discarded.

Disinfecting Procedure

The transfer of maggots and pupae was facilitated by the use of a modified spatula (Fig. 1*b*).

* Also known as NAIDM. A product of the Ralston-Purina Co., St. Louis, Missouri, consisting of wheat bran (33 per cent), alfalfa meal (26.7 per cent) and brewers' grain (40.0 per cent).

† These were generously supplied as lyophilized cultures by the Bacteriology Laboratories of the Illinois State Department of Public Health.

‡ Henceforth, though not explicitly stated, it is understood that materials used in all bacteriological procedures, *e.g.*, rinse water, grinding tubes, sand, etc., are sterile.

§ Beef-heart infusion.

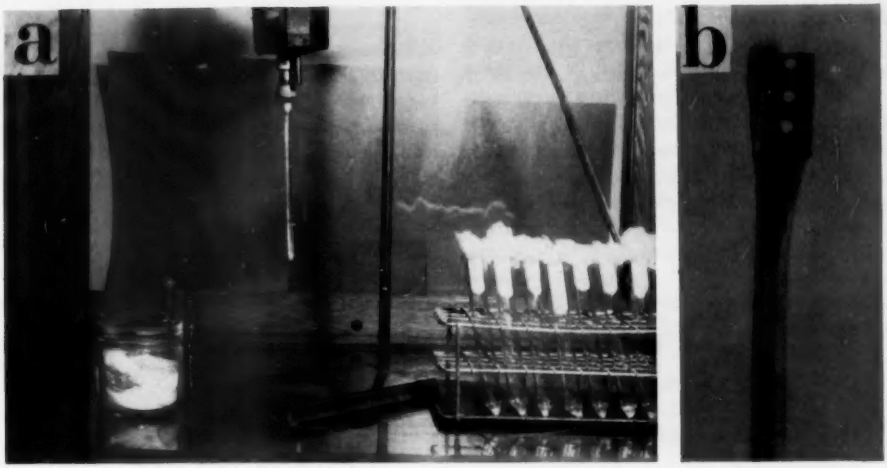

FIG. 1a. Interior of hood containing alcohol lamp, grinding apparatus and grinding tubes. b. Spatula for transferring larvae and pupae.

TABLE 1

Survival of Salmonella paratyphi *B in* 3rd *instar maggots and in emerging adults breeding in nonsterile larval medium to which the organism has been added*

Experiment	Number of maggots infected	Maggots infected (%)	Number of adults infected	Adults infected (%)
1	0/25	0	10/14	71
2	10/20	50	2/15	13
3	0/14	0	0/58	0
4	5/7	71	5/18	28
5	0/7	0	0/6	0
6	7/7	100	2/3	67

TABLE 2

Survival of Salmonella typhi *in* 3rd *instar maggots and in emerging adults breeding in nonsterile larval medium to which the organism has been added*

Experiment	Number of maggots infected	Maggots infected (%)	Number of adults infected	Adults infected (%)
1	1/8	13	0/19	0
2	1/28	4	0/13	0
3	0/28	0	0/4	0
4	0/28	0	0/15	0
5	0/28	0	0/7	0

TABLE 3

Survival of Shigella flexneri *IIa in* 3rd *instar maggots and in emerging adults breeding in nonsterile larval medium to which the organism has been added*

Experiment	Number of maggots infected	Maggots infected (%)	Number of adults infected	Adults infected (%)
1	5/12	42	0/7	0
2	0/10	0	0/10	0
3	4/15	27	0/14	0
4	2/20	10	0/17	0

During an experiment, igniting the alcohol on the spatula once or twice rendered the instrument sterile but not injuriously hot. A sample of larvae or prepupae was removed from the medium, rinsed twice and placed in a test tube containing White's solution* (10 maggots per 10 ml). After 15 minutes of immersion, the test tubes were placed in an ice bath for an additional 5 minutes. The maggots were then collectively rinsed 6 times in cold water, and immersed individually in tubes of cold thioglycollate broth for 15 to 20 minutes. Cold solutions immobilized the specimens, thereby lessening the chance of recontamination from either end of the maggots' guts. Each specimen was then transferred to a grinding tube and the thioglycollate was incubated. This treatment gave 90 to 100 per cent

* Mercuric chloride, 0.25 gm; sodium chloride, 6.50 gm; hydrochloric acid, 1.25 ml; ethyl alcohol 250 ml; distilled water, 750 ml.

successful disinfections. Pupae were disinfected in White's solution for 20 minutes, rinsed 6 times and either removed from or left in the pupa case. Since White's solution reduces the proportion of emerging adults, we used a 1:500 aqueous $HgCl_2$ solution and obtained a higher percentage of eclosions. Newly emerged adults were processed without washing or disinfection.

Grinding Procedure

The grinding apparatus is housed in a hood equipped with an ultraviolet light and an alcohol lamp (Fig. 1*a*). The grinder is a variable speed motor stirrer to which is attached a Pyrex glass rod. The rod is sterilized by flaming in alcohol. Grinding tubes are 125 × 18-mm Pyrex glass tubes with the butts evaginated as shown. The

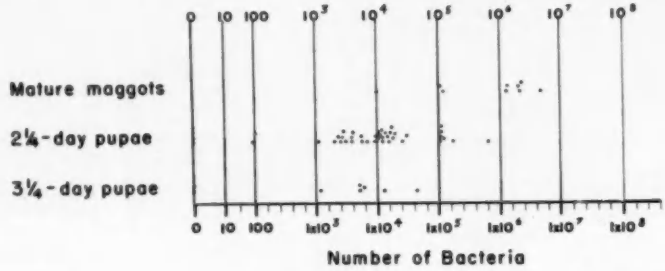

FIG. 2. Survival of *Salmonella enteritidis* in maggots and pupae reared aseptically. Each dot represents the bacterial count of a specimen, and is the average of two replicates.

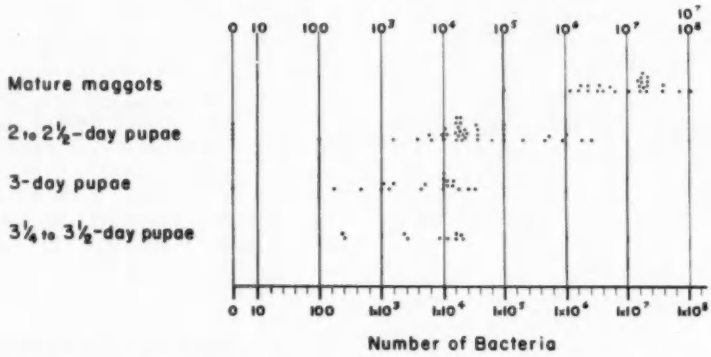
Mature maggots
2 to 2½-day pupae
3-day pupae
3¼ to 3½-day pupae
0 10 100 10³ 10⁴ 10⁵ 10⁶ 10⁷ 10⁸
0 10 100 1x10³ 1x10⁴ 1x10⁵ 1x10⁶ 1x10⁷ 1x10⁸
Number of Bacteria

FIG. 3. Survival of *Salmonella paratyphi* B in maggots and pupae reared aseptically.

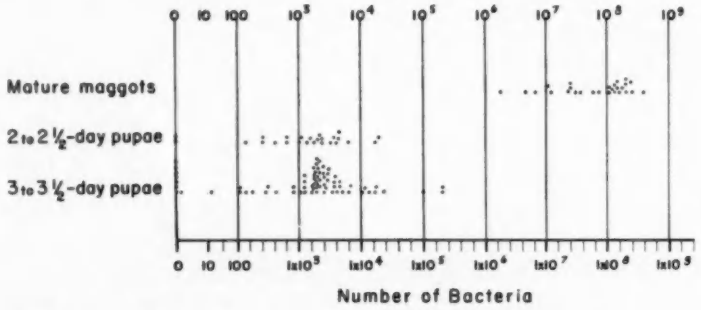

FIG. 4. Survival of *Salmonella typhi* in maggots and pupae reared aseptically.

specimen was ground in a tube containing a pinch of sand and 0.1 ml. of a 0.9 per cent saline solution. After grinding, 0.9 ml. of saline was added to the tube. The homogenates were kept cold until dilutions were made.

RESULTS

The percentage survivals of the pathogens in maggots and adults breeding in a contaminated larval medium are summarized in Tables 1, 2 and 3. *S. paratyphi* B was recovered from a variable percentage of adults, ranging from 0 to 71 per cent. In 3 of 6 experiments, a larger percentage of larvae than adults were infected. In 3 experiments, we were unable to recover the test organism from larvae, though in one such case, adults proved to be infected. *S. typhi* and *S. flexneri* were recovered in smaller percentages from larvae and not at all from adults.

The results of experiments with single species of bacteria are shown in Figures 2, 3, 4 and 5. Mature maggots typically harbor 10^7 organisms while the counts of pupae from the second day until emergence are stabilized at 10^9 and 10^8. A few pupae become sterile or retain relatively small numbers of bacteria. All 4 species undergo comparable reduction in numbers and typically survive pupation.

In another experiment, approximately equal numbers of *S. enteritidis* and *Proteus rettgeri* were introduced into the sterile larval medium. No determinations were made of the proportionate survival of each species. The results indicate, however, that dual infection is quantitatively the same as infection with a single species of bacteria (Fig. 6).

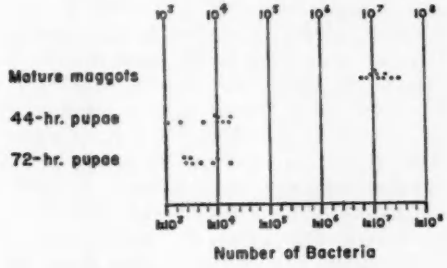

FIG. 5. Survival of *Shigella flexneri* in maggots and pupae reared aseptically.

DISCUSSION

Before pupation occurs, the majority of maggots reared in a normally contaminated medium to which *S. flexneri* or *S. typhi* have been added no longer retain these organisms. Indeed, the recovery rate for these species and *S. paratyphi* B is surprisingly low considering how recently the organisms have been added to the medium. Ledingham[6] failed to recover typhoid organisms from housefly maggots soon after introducing a broth culture into a contaminated larval medium. In breeding the larvae of *Sarcophagula* sp. in feces contaminated with *S. typhi*, *Serratia marcescens* or *Staphylococcus aureus*, Nicholls[8] found that these species rapidly disappeared from the larvae when they were removed from their infected surroundings. Melnick and Penner[7] reported early 3rd instar larvae of *Phaenicia*

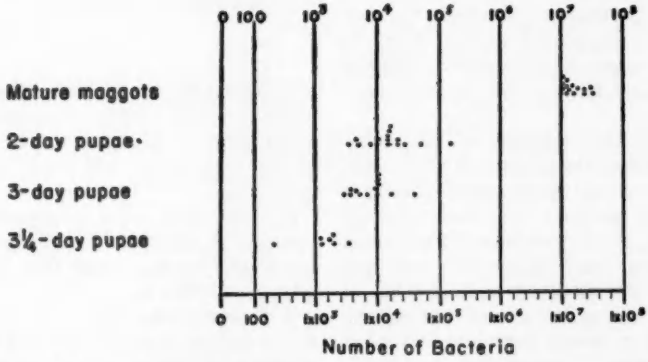

FIG. 6. Number of bacteria surviving in maggots and pupae reared solely with *Salmonella enteritidis* and *Proteus rettgeri*.

TABLE 4

Survival of S. typhi *in sterilized larval medium*

Flask	Number* of organisms per ml at:		
	Zero time	2 days	4 days
A (with maggots).....	2.5×10^7	2.6×10^{10}	8.0×10^9
B (without maggots)...	2.5×10^7	2.2×10^{10}	1.4×10^{10}

* Each figure is an average of 3 replicates.

sericata (Meig.) infected with poliomyelitis or Coxsackie virus retained only minimal amounts of the virus 1 and 3 days after feeding, and none of the adults was infected.

Our findings confirm earlier conclusions that typhoid and dysentery bacteria ingested by maggots under these conditions do not persist to the adult stage. Contrary to earlier findings, *S. paratyphi* B does persist, and in this respect, it is similar to *S. enteritidis* and *S. pullorum*. It is noteworthy that the infection rate in adults is lower than in maggots, indicating that at some stage between maggot and adult, at least partial destruction of the bacterial population occurs.

Normally, the maggot flourishes in a fermenting medium, ingesting large numbers of bacteria with, and perhaps as, its food. In this regard, the complex, yet unknown interactions of the so-called normal maggot flora and the pathogens, in both the larval medium and the larval gut, cannot be ignored. This flora may exert an antibiotic effect on the pathogens, while such host factors as pH and bacteriophage may contribute to an unfavorable milieu. By employing sterile maggots and eliminating, to a considerable extent, the effects of a competing flora, we focussed more sharply on the insect as host.

To determine the suitability of this axenic method we studied the persistence of *S. typhi* in the sterilized larval medium, with and without the presence of maggots. The sterile medium contained in a 125-ml Erlenmeyer flask received 5 ml of a 24-hour broth culture of *S. typhi* and approximately 200 sterilized eggs. Ten ml of distilled water were added to make the medium sufficiently wet to permit fluid to collect when the flask was tilted. Another flask was identically prepared but received no eggs. Three samples of fluid were plated from each flask after the larval medium was thoroughly mixed. These samples represented zero time. The flasks were then placed at 36°C and three additional samples from each flask were taken at the end of 2 and 4 days. The results are shown in Table 4. In the absence of competing organisms, *S. typhi* more than held its own during the 4-day period, and probably underwent some multiplication. The presence of maggots in the medium apparently did not effect the survival of *S. typhi*.

We were now assured that large numbers of the test organism were present during the growth and feeding phase of the maggots. The axenic method enabled us to quantitatively follow the fate of a single species of pathogen in the larva and pupa. Such data provide a picture of the extent of infection in these stages which mere positive or negative survival data cannot do. Our evidence clearly indicates pathogens are able to survive pupation in the absence of a competing flora. It seems reasonable to suggest, therefore, that the complete elimination of *S. typhi* and *S. flexneri* from flies breeding in contaminated media is most likely a consequence of competitive inhibition by the indigenous flora. Though we emphasize the ecology of the larval medium, we do not wish to underestimate the influence of fly factors on bacterial survival. We have noted a marked and consistent decrement between the larval and pupal flora. The causes of this decline must be sought in host phenomena preceding and accompanying metamorphosis. An analysis of these factors will be presented in subsequent papers.

SUMMARY

1. *Salmonella typhi* and *Shigella flexneri*, introduced into CSMA, a normally contaminated larval medium, were recovered infrequently from larvae and not at all from adult houseflies.

2. Under the same conditions, *Salmonella paratyphi* B was recovered more frequently from larvae and adults, though from a smaller percentage of adults than larvae.

3. *S. typhi*, when introduced into a sterilized larval medium, probably underwent some multiplication, whether or not maggots were present.

4. Each of the above species, including *Salmonella enteritidis*, when introduced into a sterilized larval medium, was routinely recovered from larvae and pupae of various ages. Mature maggots typically harbored about 10^7 organisms. The counts of pupae from the second day until emergence were stabilized at 10^3 to 10^4. A small number of pupae were sterile or retained relatively few bacteria.

5. An explanation based on interspecific competition in the larval medium is suggested for the differential survival of these pathogens in fly stages breeding under natural conditions.

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PERSISTENCE OF BACTERIA IN THE DEVELOPMENTAL STAGES OF THE HOUSEFLY

II. Quantitative Study of the Host-Contaminant Relationship in Flies Breeding Under Natural Conditions*

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The transmission of *Salmonella typhi* or *Shigella flexneri* from the larva to the adult housefly is consistently unsuccessful if the pathogen is introduced into a contaminated larval medium. Under the same conditions, limited transmission occurs with an organism such as *Salmonella enteritidis*. Under axenic conditions, wherein all biological interaction is reduced to that between a single species of bacteria and the fly host, transmission of *Salmonella* and *Shigella* is readily accomplished.[4] The differential survival of these species in a contaminated milieu, and their obvious decline in number around pupation, even under axenic conditions, prompted further study of the pattern of bacterial survival in the developing fly.

In the summer of 1957, we undertook field collections of maggots, prepupae and pupae from various breeding sites located in Cook County, outside of Chicago. The purpose of these field studies was to validate the reliability of our laboratory findings in terms of actual breeding conditions as they exist in a community. In applying our previous approach to a comparative study of bacterial survival in flies breeding in various natural media we hoped to gain information on the following:

1. Determination of the bacterial species which successfully survive pupation in both laboratory and natural media.

2. Pattern of bacterial survival in larva, pupa and adult bred in laboratory medium as compared with those bred in a variety of natural media.

3. Whether certain breeding materials produce flies with greater than usual populations of bacteria.

* This study was supported by a grant from the University of Illinois Research Board and, in part, by a contract with the Illinois State Department of Public Health. The author is grateful to Dr. Howard J. Shaughnessy, Deputy Director of the Illinois State Department of Public Health, for making funds available for the field studies herein reported.

The author is happy to acknowledge the contribution of Miss Esther Cooksey of Chicago in the preparation of the charts.

MATERIALS AND METHODS

The availability of abundant samples of each stage enabled us to follow the progress of infection far more closely than had been practicable in the single-species studies. We have therefore included prepupae and several additional pupa stages, as well as newly emerged adults. A detailed description of the entomological and bacteriological materials and methods is given in Part I of this series, including techniques for the disinfection, grinding and plating of specimens, and criteria for the identification of the several fly stages. One difference in method should, however, be noted. In the single-species experiments reported previously, pupae were removed from their puparia and ground, and the grinding rod was not rinsed. In this study, pupae were ground within their puparia and, after grinding, 0.9 ml. of sterile* saline was used to wash the residue on the grinding rod into the tube. The latter innovation undoubtedly improved the recovery of bacteria.

The fly-breeding media surveyed in this study were laboratory CSMA, human feces, garbage and horse manure. The human feces were located in a privy built level with the ground. Heavy rains during the period of sampling rendered the privy contents semifluid. Among the other insects present in the medium were larvae of of *Tubifera tenax* (Linn.) and *Fannia scalaris* (Fabr.) A garbage dump supported enormous populations of blowflies but relatively few houseflies. A considerable number of housefly larvae were located within some empty shrimp cartons buried in a pile of wood shavings. The cartons

* Henceforth, though not explicitly stated, it is understood that materials used in all bacteriological procedures, *e.g.*, rinse water, saline, grinding tubes, sand, etc., are sterile.

were quite damp and retained the odor and some fragments of shrimp. The horse manure was piled daily in the yard of a riding academy and represented an accumulation of considerable size and age. Maggots and prepupae were returned to the laboratory in the medium in which they were collected and processed within the next few hours. Daily collections were made in order to study these two stages. White pupae collected in the field were thoroughly washed, disinfected in the laboratory and kept together in small Petri dishes or singly in corked test tubes in a chamber with a relative humidity of 94 per cent. Room temperature during the period of this study fluctuated around 32°C. Pupae were removed and processed at intervals, at which time a few specimens were dissected to note the development of the sample. The age of pupae given in the charts, however, is the actual and not the developmental age. Emerging adults were immobilized in the refrigerator, transferred to a grinding tube and processed.

TABLE 1

Species of bacteria from newly emerged flies breeding in horse manure

Fly specimen	Species of bacteria
1	*Proteus vulgaris, Proteus mirabilis, Aerobacter aerogenes, Escherichia freundii*
2	*P. vulgaris, A. aerogenes, E. freundii*
3	*A. aerogenes*
4	*P. mirabilis, A. aerogenes, E. freundii*
5	*P. vulgaris, A. aerogenes, E. freundii*
6	*A. aerogenes*
7	*P. vulgaris, A. aerogenes, E. freundii*
8	*A. aerogenes, E. freundii*
9	*P. mirabilis, E. freundii*

RESULTS AND DISCUSSION

To determine the species of bacteria which survive the pupation process, we used pupae obtained from the regular CSMA and from horse manure. The puparia were disinfected and

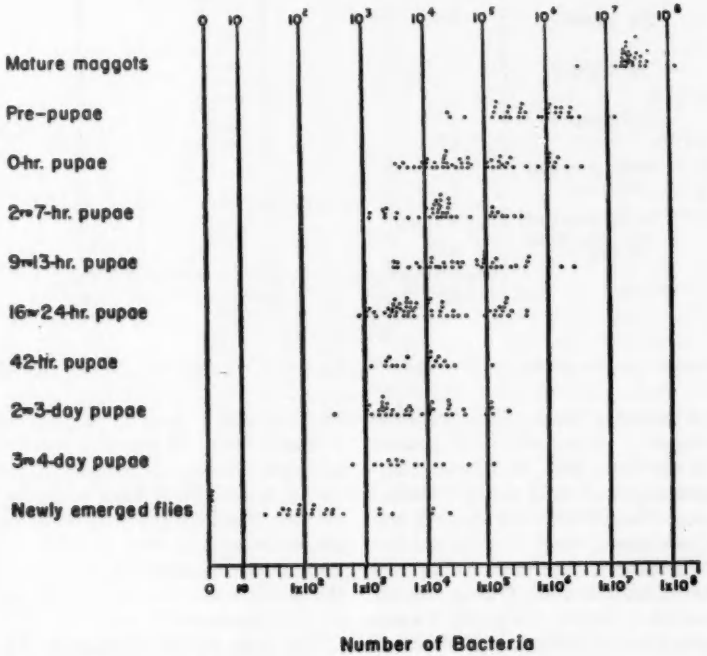

FIG. 1. Quantitative survival of bacteria in mature maggots, prepupae, pupae and newly emerged adults breeding in CSMA. Each dot represents a single specimen and is the average of two replicates.

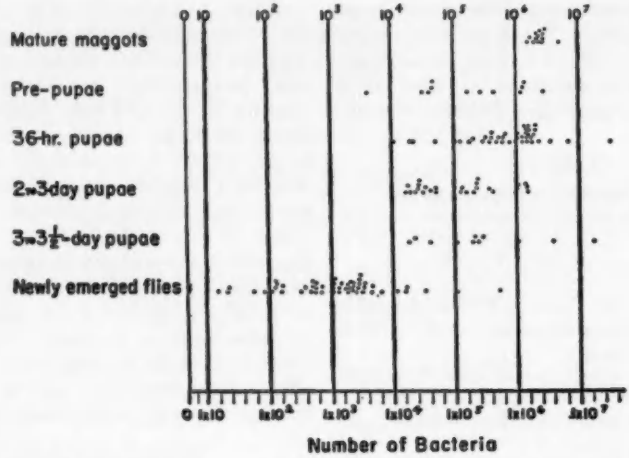

FIG. 2. Persistence of bacteria in fly stages breeding under natural conditions in horse manure.

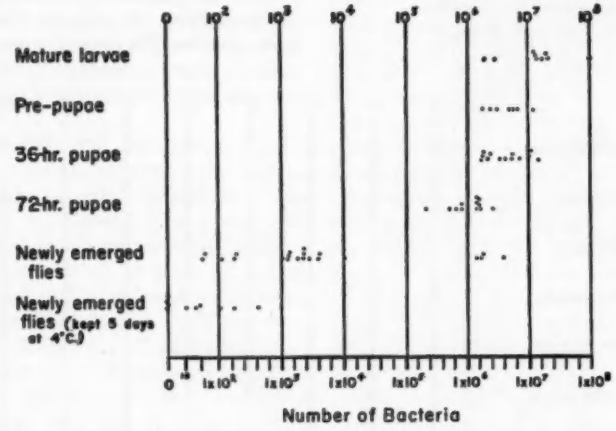

FIG. 3. Persistence of bacteria in fly stages breeding under natural conditions in garbage.

isolated, and emerging adults were processed without treatment. Identification of bacteria was based on the Gram stain, motility and biochemical reactions, as given in *Bergey's Manual of Determinative Bacteriology*.[1] No attempt was made to culture yeasts, fungi or obligate anaerobes. The species recovered from laboratory-bred flies were *Pseudomonas* sp., *Proteus vulgaris*, *Proteus mirabilis*, *Proteus morganii*, *Proteus rettgeri*, *Escherichia coli*, *Escherichia freundii* and *Aerobacter aerogenes*. Table 1 is a census of species present in emerging adults bred in manure. It is notable that some flies retained only a single species of bacteria whereas the majority of flies harbored an assortment of species. These species are widely distributed saprophytes present in the feed and feces of horses and other animals. The majority have often been isolated from the housefly itself and have come to be considered its normal flora. They are undoubtedly the predominant flora of the larval medium used in the laboratory.

The data of 10 experiments are pooled in Figure 1 which summarizes the quantitative survival of bacteria in mature maggots, prepupae, pupae and newly emerged adults breeding

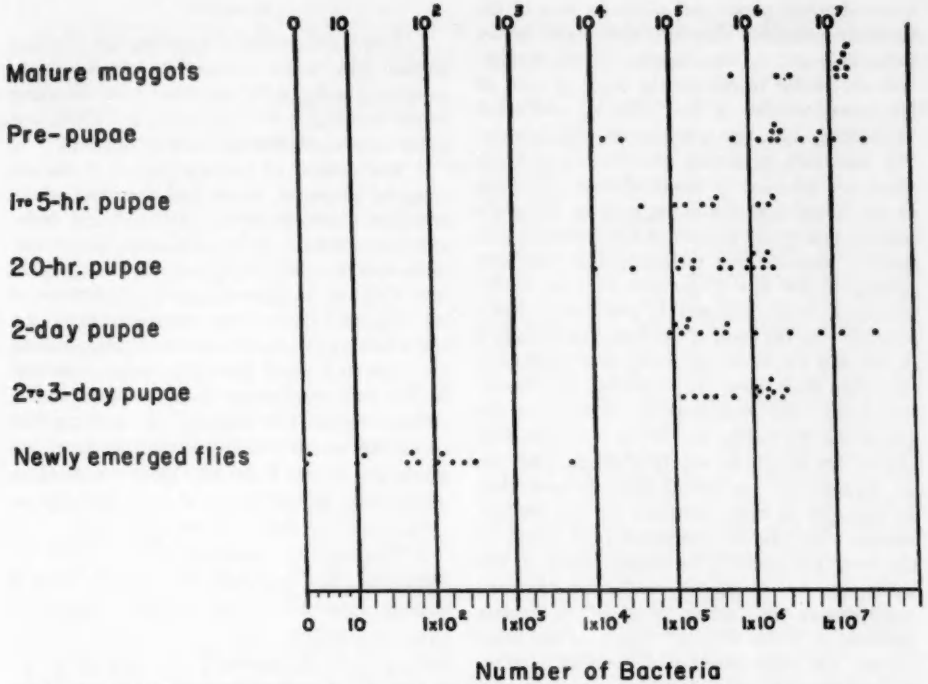

FIG. 4. Persistence of bacteria in fly stages breeding under natural conditions in privy contents.

in CSMA. Mature maggots support populations of 10^7 bacteria, as previously observed in the single-species infections. The uniformity of the bacterial count is typical of the actively feeding larva. In the prepupa the count falls to 10^6 and 10^5, this stage exhibiting far more variability. Since the prepupal stage lasts about a day, the number of bacteria in the white pupa serves both as an indicator of the terminal count of prepupae and the initial count of pupae. The commencement of pupation is characterized by counts of 10^4 and 10^5, with some variation at the upper and lower limits. This range of infection is maintained during the 4-day period of metamorphosis. Emerging flies show a considerable reduction, with the majority at 10^2, but varying from 0 to 10^6. A comparison of the data obtained from manure, privy contents and garbage substantiates the over-all pattern noted above (Figs. 2, 3 and 4). Again two declines in the bacterial population may be noted: in prepupae and in emerging adults. The latter typically have counts between 10^2 and 10^3. Further comparison discloses a broader range of pupal infection in CSMA. This is probably the result of more extensive sampling and is perhaps a truer expression of the extent of variability in this stage. This variability originates with prepupae which differ in their ability to retain bacteria ingested by the maggot. The differences among the data for the various breeding media are interpreted as the natural variability in the host-contaminant association and not as a measure of significant differences produced by any specific medium. Whatever the medium, adults emerged with about the same number of bacteria.

Comparison of infection with single species of *Salmonella* or *Shigella*, dual infection with *S. enteritidis* and *P. rettgeri*, and multiple infection with a saprophytic flora leads us to conclude that all these organisms respond similarly to the same host factors, as evidenced by the essentially uniform pattern of survival in every case.* In this context, we may note the occur-

* The nature and mode of action of some of these host factors will be discussed in a forthcoming paper.

rence of sterile pupae and adults in each of the breeding situations described above and in the previous paper. In summarizing his own studies, Graham-Smith[3] represents the point of view of his contemporaries in the following statement concerning the fly-contaminant relationship: "Of non-spore producing organisms only those which are adapted to the conditions prevailing in the intestine of the larva, such as Morgan's bacillus and certain nonlactose fermenting bacilli, survive through the metamorphosis and are present in the flies. Organisms such as *B. typhosus*, *B. enteritidis*, and *B. prodigiosus* rarely survive." On the basis of the evidence presented in this and the preceding study, it is considered doubtful that these saprophytes are better suited than the pathogens for survival in the gut of the fly during any of its developmental stages. The significant ability of the saprophytes to multiply in the larval medium and thus be ingested in large numbers by the maggot assures their presence in subsequent stages of the host. The inability or limited ability of the pathogens to propagate under competitive conditions in the various larval media likewise prevents or limits their retention by the host. Among the many studies of bacterial antagonism pertinent to our discussion, we need only mention two. Topley and Fielden[6] studied the succession of species in broth cultures incubated over considerable periods. They found in one such experiment that *S. typhi*, *Salmonella paratyphi* A, *Shigella dysenteriae* and *S. flexneri* could not be recovered in the initial subculturing which occurred a few days after the organisms were inoculated into the medium. Such species as *P. vulgaris*, *P. morganii* and *E. coli* were predominant, though not simultaneously. More recently, Freter[2] demonstrated, in vivo, the inhibition of *S. flexneri* and *Vibrio comma* by the normal enteric flora of mice and guinea pigs, and also by a streptomycin-resistant *E. coli*.

The need to breed in fermenting media defines the extent of the maggot-contaminant association, for the maggot is nutritionally dependent on the indigenous flora. Without these microorganisms or their products the first instar maggot cannot grow.[5] It thus appears that enteric pathogens are destroyed in the very environment required by maggots for their development.

SUMMARY

1. Listed are species of bacteria, the so-called normal flora, which successfully survive metamorphosis and can be recovered from emerging adults breeding in the laboratory in CSMA, and under natural conditions in horse manure.

2. The number of bacteria present in mature maggots, prepupae, pupae and emerging adults breeding in the laboratory in CSMA and under natural conditions in horse manure, privy contents and in garbage is presented in graphic form. Mature maggots support populations of 10^7 bacteria. During the prepupal stage, the count falls to 10^5 and 10^6. At the commencement of pupation counts generally range from 10^6 to 10^5, and this range is maintained during metamorphosis. The majority of emerging flies retain 10^2 or 10^3 bacteria, whereas some are sterile and others have 10^6. Thus two declines consistently appear in the survival pattern: in the prepupa and the newly emerged adult.

3. Single-species infection with *Shigella* or *Salmonella*, dual infection and infection with a multiple saprophytic flora exhibit a pattern of bacterial survival which is essentially similar in all cases. On the basis of the evidence presented in this and the preceding study, it is considered doubtful that the normal flora are better suited than the pathogens for survival in the gut of the developing fly.

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AN INTESTINAL PARASITE SURVEY ON RONGELAP ATOLL IN THE MARSHALL ISLANDS

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In March 1954, 84 Marshallese inhabiting the Atoll of Rongelap were accidentally irradiated with fallout following the experimental explosion of a large nuclear device. They were quickly evacuated to other atolls in the Marshall Islands due to radioactive contamination of their home atoll. In 1957 their island was considered safe for habitation and a new permanent village was constructed for them. They were returned there in July of that year. In addition, a group of Rongelap people who were not exposed to radiation because they were away from the island at the time of the accident, returned to live with their fellow islanders on Rongelap. This group has served as a comparison population for examinations conducted during the past two years.

Extensive medical examinations have been carried out on these people since their exposure.[2-6]* In order to attempt to explain the generally high incidence of eosinophilia in these people,[5] and in view of the lack of reliable parasitological information for the area, an intestinal parasite survey was conducted as part of the examinations carried out in March 1958, four years after exposure to radiation. Sixty-nine exposed and 112 nonexposed individuals were examined in the intestinal parasite portion of this examination.

Because general information concerning the island is relatively sparse, we propose to present here, in addition to parasitological information, some background material so that the reader may have an idea of the environment in which the Marshall Islanders live. Most of this material has been abstracted from "Military Geography of the Northern Marshalls"[7] and much of it was confirmed by personal observations of the authors.

* These surveys are carried out by the Brookhaven National Laboratory, with participants from other organizations, and are under the direction of Dr. Robert A. Conard of Brookhaven National Laboratory. They are sponsored by the Atomic Energy Commission with the collaboration of the Department of Defense.

GENERAL BACKGROUND

The Marshall Islands constitute a group of low coral atolls in the Pacific Ocean about 2400 miles west-southwest of the Hawaiian Islands. They extend north of the equator from 4° to 15°, and in an east-west direction from 161° E. to 172° E. Rongelap Atoll is one of the most northern of the group with approximate coordinates of 11° N. by 166° E. The total area of this atoll is 426 sq. miles of which only 2.5 sq. miles are land. The population in 1954, when the fallout occurred, was about 100. At the time of this survey, the population was not known accurately but was probably over 200.

Climate

Rongelap Atoll has a tropical marine climate characterized by uniform temperature, high humidity, cloudiness and wind. The mean annual temperature is remarkably constant at about 80°F with mean monthly temperatures not varying more than 2°. The sunlight is intense and the heat is uncomfortable in sunny places sheltered from the trade wind. Mean relative humidity is about 80 per cent and is comparatively uniform throughout the year.

Most of the rain falls in moderate to heavy showers of short duration during late summer and early fall. If a hurricane develops in the area, several days of almost continuous rain may occur. Annual mean rainfall in Rongelap is about 65 inches but there may be great variation from year to year. Although this does not represent a dry climate as understood in continental regions, the special soil conditions on the atolls result in a condition of semiaridity with such levels of precipitation.

Terrain

The usual arrangement of an atoll is a reef of varying width, irregularly ring shaped, enclosing a basin of water called a lagoon. Most of the reef is at or just below water level, but at intervals, low vegetated islets of all sizes and shapes

FIG. 1. Rongelap islet as seen from the lagoon

FIG. 2. Aluminum roof with downspouts leading into concrete cistern for catching rain water.

may occur. Rongelap Atoll is approximately 20 by 35 miles in its greatest dimensions, and Rongelap islet, where the survey was performed, is the largest in the atoll. The islet is several miles long but only a fraction of a mile wide. Its highest prominence is probably less than 15 feet above sea level (Fig. 1).

Drainage and Water Supply

The "soil" on the islet is composed basically of loose organic limestone debris accumulated on the reef surface. The material is coarse and porous so that drainage by percolation through the ground is almost instantaneous. The amount of humus from decaying vegetation varies from place to place but nowhere is it more than a thin layer. There is normally no running or standing surface water. The only naturally occurring fresh water is rainfall which is caught on the aluminum roofs of houses and drained into cisterns (Fig. 2). Ordinarily, there exist in these coral islets, bodies of subsurface fresh water known as Ghyben-Herzberg lenses, but, so far as we know, this source of water was not used for drinking on Rongelap islet. There did appear to be one well or pit where clothes were washed but, for the most part, cistern water was used for that purpose. Stored rain water is used without chemical or other treatment.

Flora and Fauna

In general, the species of plants making up the vegetation are few in number. Coconut plantations form the dominant plant life on Rongelap but, in addition, forests of other types of trees or tall shrubs exist. Grasses of various types are also found. The canopy in these forests is not thick but considerable shade is provided. An important physiological characteristic of the plants is a high salt tolerance. The windward sides of the islet are exposed to continual drenching with salt spray, and the size of Rongelap islet is such that probably all of the soil is exposed to salt washed down by rainwater from the canopy.

In keeping with the generally limited biological potential of the islets, the numbers and types of land animals are small. The only mammals (besides man) seen on Rongelap were pigs, dogs, cats and wild rats. The three domestic species are not numerous. The pigs forage in and away from the village, whereas the cats and dogs stay around the houses. The wild rat is a small species which lives in the brush away from human habitation. It invades houses in search of food but lives principally on coconuts. The only other domestic animals on Rongelap at the time of the survey were chickens. These ranged around the village.

The other obvious land animals on Rongelap are arthropods, of which the coconut crab is probably the largest. In general, insects, except for flies, were hardly noticed. One was much less conscious of them while he walked through the forests on Rongelap than in a wooded area practically anywhere in the United States. Houseflies, however, were common in the village. They were persistent in lighting on bare skin and were a distinct annoyance. Unlike houseflies in the States, which can be caught by flypaper hanging from the ceiling, these flies almost never flew above table level except to light on an individual.

In contrast to the condition on land, the sea and lagoon teemed with living things. Many varieties of fish, crustacea and mollusks were

to be found, and some of them were important food items for the islanders.

Culture and Living Conditions

The Marshallese are a Micronesian people (Figs. 3, 4 and 5). They are brown, less than medium American height, of unknown origin but speak a language with Indonesian relationships. Their hair is black and generally straight or curly but not kinky. They prefer to live in villages rather than in isolation. On Rongelap the village was situated on the lagoon side of the islet (Fig. 6). The houses were new, having been recently provided by the American government as the trust power. The houses are mostly raised on stilts, leaving a space about 3 feet high under the floor (Fig. 7). Much of the activity around the house takes place in this shaded, dry area, the house itself serves primarily for sleeping purposes. Sanitary facilities consist of pit privies similar to those in rural areas in the States. These are provided for each house or small group of houses.

FIG. 3. Rongelappese girls and a boy

FIG. 4. Rongelappese boys

FIG. 5. Rongelappese adults

FIG. 6. Village on Rongelap islet, bordering the lagoon.

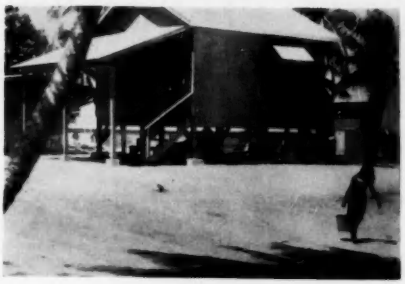

FIG. 7. Typical new house showing construction on stilts.

Family relationships are difficult to define by the American system. Most inhabitants of the islet are related to each other and families frequently visit and eat with one another. It was common to distribute 6 or 7 cartons for stool collections to a single household. There were many children and young people on Rongelap. During the period of the survey, school was not in session and the children generally played in large groups. Older children commonly

FIG. 8. Children at play; note very young child in the group.

took care of the younger ones, including the toddlers (Fig. 8).

The Rongelapese dress as the Americans do in the same climate, with the exception that a higher percentage of them go barefoot. They eat flour, rice and canned foods in addition to fish, clams and other mollusks. Local vegetable foods are limited to coconuts, taro tubers, breadfruit and pandanus fruit. The islanders appear to be well fed and generally are in good health. The normal pattern of activity of these people, which is concerned with coconut cultivation and fishing, had been disrupted by the events of the past few years. At the time of the survey they had not yet entirely gone back to their usual activities.

Although, as mentioned above, privies are available around the houses in the village, it is probable that defecation is common in the brush by both children and adults. The practice of morning defecation on the beaches below the high-tide line appears to have disappeared among the Rongelapese.

MATERIALS AND METHODS

The procedures used for stool examinations were direct examinations of saline mounts, using the Beaver[1] method for standardizing the preparations, and formalin-ether concentrations.[5] One stool specimen was examined per person. Cartons were distributed in the evening with the request that specimens be brought in the next day at any time. As a result, most specimens were examined within an hour after being passed. Egg counts were made on the Beaver preparations.

RESULTS

Infections found in the two main groups examined are tabulated in Table 1. For most parasites the incidence in exposed and nonexposed populations was close enough to be considered the same. There were more infections with small race *Entamoeba histolytica* and with hookworm in the nonexposed group, and more infections with *Trichomonas hominis* in the exposed group. For the three major pathogens found the over-all infection rates were: *E. histolytica*, 18.2%; hookworm, 5.5%; *Trichuris trichiura*, 34.3%.

Of the 69 exposed individuals, 40 had eosinophile counts of 5% or more, and 29 had counts of less than 5%. Among the nonexposed individuals, there were 60 cases on whom both stool examinations and eosinophile counts were avail-

TABLE 1

Relationship of radiation exposure to infection with intestinal parasites

Organism	Exposed (69 cases)	Nonexposed (112 cases)	Totals (181)
Entamoeba histolytica	14 (20.3)*	19 (16.9)	33 (18.2)
Entamoeba histolytica (small race)	2 (2.9)	10 (8.9)	12 (6.6)
Entamoeba coli	25 (36.2)	35 (31.3)	60 (33.1)
Endolimax nana	14 (20.3)	35 (31.3)	49 (27.1)
Iodamoeba butschlii	—	3 (2.7)	3 (1.7)
Giardia lamblia	5 (7.2)	9 (8.0)	14 (7.7)
Chilomastix mesnili	4 (5.8)	4 (3.6)	8 (4.4)
Trichomonas hominis	24 (34.8)	30 (26.8)	54 (29.9)
Hookworm	2 (2.9)	8 (7.1)	10 (5.5)
Trichuris trichiura	21 (30.4)	41 (36.7)	62 (34.3)
No parasites	11 (15.9)	30 (26.8)	41 (22.7)

* The figures in parentheses are per cent.

TABLE 2

Relationship of eosinophilia to infection with intestinal helminths

Organism	Exposed population		Nonexposed population		Combined population	
	Eosinophilia (40 cases)	No eosinophilia (29 cases)	Eosinophilia (34 cases)	No eosinophilia (26 cases)	Eosinophilia (74 cases)	No eosinophilia (55 cases)
T. trichiura	14 (35.0)*	7 (24.1)	17 (50.0)	7 (26.9)	31 (41.9)	14 (25.5)
Hookworm	1 (2.5)	1 (3.4)	5 (14.7)	2 (7.7)	6 (8.1)	3 (5.5)
No helminths	26 (65.0)	21 (72.4)	13 (38.2)	19 (73.1)	39 (52.7)	40 (72.7)

* The figures in parentheses are per cent.

TABLE 3

*Relationship of age of individuals to infection with intestinal parasites**

Organism	Age of individuals (yrs)				
	1-5 (43 cases)	6-12 (30 cases)	13-20 (15 cases)	21-50 (56 cases)	51 and older (29 cases)
E. histolytica	2 (4.6)†	7 (23.3)	2 (13.3)	15 (26.8)	5 (17.2)
E. coli	10 (23.3)	10 (33.3)	5 (33.3)	20 (35.7)	14 (48.3)
E. nana	8 (18.6)	4 (13.3)	7 (46.7)	18 (32.1)	9 (31.0)
G. lamblia	8 (18.6)	2 (6.7)	0	1 (1.8)	1 (3.4)
T. hominis	13 (30.2)	11 (36.6)	3 (20.0)	17 (30.4)	8 (27.6)
Hookworm	1 (2.3)	1 (3.3)	1 (6.7)	7 (12.5)	0
T. trichiura	13 (30.2)	19 (63.3)	11 (73.3)	9 (16.1)	9 (31.0)
No parasites	17 (39.5)	3 (10.0)	2 (13.3)	11 (19.6)	5 (17.2)

* Eight individuals whose ages were unknown are omitted from this table.

† Figures in parentheses are per cent.

able. Of these, 34 showed eosinophilia, 26 did not. When the incidence of *Trichuris* was determined in these groups, it was found that in both exposed and nonexposed populations more of the eosinophilia cases had *Trichuris* infections than those with no eosinophilia (Table 2). However, about half of the cases with eosinophilia showed no helminth infections at all.

In 11 of 17 *Trichuris* infections only one egg was found in the Beaver preparation. The number of eggs in the remaining 6 ranged from 2 to 6. Similarly, the hookworm burden was low.

Because infection rates for both exposed and nonexposed groups were similar, the following analyses are based on pooled results for both groups.

Table 3 presents the age distribution of infections found. *E. histolytica* was found in 4.6% of 43 children 5 years old or younger. In the 6 to 12 year group, detected infections went up to 23.3%. The highest incidence, 26.8%, was found among adults in the 21 to 50 age group. The other two intestinal amebas, *Entamoeba coli* and *Endolimax nana*, occurred much more frequently in the youngest age group, 23.3 and 18.6%, respectively, and both showed increases among the older individuals. Among flagellates, the *Giardia lamblia* incidence was highest in the young children and almost nonexistent after the age of 12. *T. hominis* showed a high, relatively unchanged incidence in all age groups.

Of the two helminths found, hookworm showed a steady rise in incidence with age up to 50, from 2.3 to 12.5%. No infections were found after 50. *T. trichiura* occurred in 30.2% of very young children, 5 years or less, and in 66.7% of the 6 to 20 year group. In the older age group, 21 to 50, the incidence dropped to 16.1%, rising again, after 50, to 31%.

Table 4 shows a breakdown of infections according to sex, with a further division into two groups, less or more than 13 years of age. Division into the two age groups was made on the basis that both sexes probably engaged in similar

TABLE 4

*Relationship of sex of individuals to infection with intestinal parasites**

Organism	Male		Female	
	Under 13 years (41 cases)	13 and over (49 cases)	Under 13 years (32 cases)	13 and over (51 cases)
E. histolytica	5 (12.2)†	10 (20.4)	6 (18.7)	12 (23.5)
E. coli	14 (34.1)	22 (44.9)	6 (18.7)	17 (33.3)
E. nana	6 (14.6)	20 (40.8)	6 (18.7)	14 (27.5)
G. lamblia	5 (12.2)	1 (2.0)	5 (15.6)	1 (2.0)
T. hominis	14 (34.1)	11 (22.4)	10 (31.3)	18 (35.3)
Hookworm	0	6 (12.2)	2 (6.3)	2 (3.9)
T. trichiura	18 (43.9)	10 (20.4)	14 (43.8)	19 (37.3)
No parasites	9 (22.0)	8 (16.3)	11 (34.4)	10 (19.6)

* Eight individuals whose sex was unknown are omitted from this table.

† Figures in parentheses are per cent.

pursuits up to puberty, but that afterwards their daily routines probably differed. Older males had higher or similar incidences than older females for all parasites except *T. trichiura*, of which incidence was about half as great in males. Among younger males incidences tended to be lower than among young females except for *E. coli* which was distinctly higher among the males.

The only recent information available to us on intestinal parasites in the Marshallese was from a survey performed by the Medical Department of the U.S.S. Whidbey in 1948–1950. This report is deposited at the Bishop Museum, Honolulu. Sugar flotation was used to examine the stools and this may account for the rather low incidence of protozoan infections found. In that survey individuals were examined from many atolls of the Marshall Islands, but the over-all picture for helminths was similar to our findings, namely: hookworm, 6.8%; *Trichuris*, 11.0%; and *Ascaris*, 0.3%.

DISCUSSION

Although differences in incidence of various parasites occurred in exposed and nonexposed groups, there is no convincing indication that radiation had anything to do with the variations. In most instances differences can be accounted for by sampling errors in the relatively small number of cases studied. In addition, the two groups are not precisely comparable. The nonexposed "control" group consisted of individuals who lived on different islands before joining the exposed population of Rongelap. Environmental sanitation was not necessarily the same for the two groups.

The environment on the coral atolls and the customs of the inhabitants are such that it was not expected that any trematode or cestode infections would be found. However, the complete absence of *Ascaris* in the face of a 34.3% incidence of *Trichuris* was unexpected. Life cycles of both these nematodes in the external environment are such that they are frequently found together in other areas. The history of Marshallese association with other peoples, Europeans, Japanese and Americans, makes it likely that they have been exposed to *Ascaris*. Thus, one is led to the possibility that the external environment on Rongelap Atoll may be unfavorable for *Ascaris* even though very suitable for *Trichuris*. We were not successful in obtaining stools from any of the few dogs on the island in order to check for dog ascarids. Several dried pig droppings were examined at one time and no *Ascaris* was found in them. However, in the absence of more epidemiological and experimental information, one can only guess as to the reasons for the lack of *Ascaris*. Soil moisture, salinity, pH, porosity, etc., may all play some part in this unusual picture.

The fact that half the cases with eosinophilia showed no helminthic infections at all suggests that there were other significant factors causing this blood picture besides parasitic infections. On the other hand, the greater incidence of *Trichuris* among patients with eosinophilia than among those without eosinophilia, indicates that infection with that helminth was probably

a contributing factor in the incidence of eosinophilia.

It is obvious from the high incidence of parasitism in all age groups that fecal contamination is widespread and that infections begin to be acquired early in life. Although individual Marshallese are generally neat and clean looking, their simple sanitary facilities and rural life make it likely that fecal contamination is a continuing affair. The concentration of *Giardia* in children conforms to the usual picture for the incidence of this parasite. It would be unwise to ascribe special reasons for differences in infections between the sexes. Not enough individuals were examined, differences show no simple pattern, and the unsettled living conditions of these people during the past few years undoubtedly has upset their usual daily routine.

The findings of the U.S.S. Whidbey are similar to ours in the almost complete absence of *Acsaris* and in the hookworm level. We found about 3 times as many *Trichuris*, but this may have been due to better examination facilities and opportunities. The low incidence of *Ascaris* on these Islands contrast with the high rates reported by the Whidbey for Saipan (44.2%) and Palau (16.8%). It would seem that a real biological phenomenon is indicated by the *Ascaris/Trichuris* ratio of the Marshalls versus the rest of the "nearby" islands.

SUMMARY

1. A survey is reported of intestinal parasitism occurring in 69 Marshall Islanders exposed to radioactive fall-out four years previously. This is compared with parasitic infections occurring in 112 nonexposed Marshall Islanders.

2. No significant difference was found between the two groups. The over-all rates for *Entamoeba histolytica*, *Trichuris trichiura* and hookworm were 18.2, 34.3 and 5.5 per cent, respectively. No other important parasitic pathogens were found. Infected individuals generally had low worm burdens and none of the amebiasis cases showed clinical symptoms attributable to this infection.

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TRANSPLACENTAL PASSAGE OF ANTIBODY TO WESTERN EQUINE AND ST. LOUIS ENCEPHALITIS VIRUSES*

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Western equine encephalitis (WEE) has a predilection for the very young individual.[9, 6, 8, 12] The tendency of the virus to strike this segment of the population is illustrated by Table 1, which shows that nearly three-fourths of the clinically evident (and laboratory proved) cases in California have occurred in individuals under the age of 10 years and about one-half have occurred in infants under the age of one year; in the 1952 outbreak in California 69 per cent of the infant cases were individuals under three months of age.[8, 12] This is in marked contrast to St. Louis encephalitis (SLE), which is seldom encountered in infants under one year of age (Table 1).

The reasons for this marked difference in the incidence in the very young are unknown. One explanation might lie in possible differences in infection rates produced by the two viruses, so that the relative absence of clinical infections with the SLE virus in infants under one year of age might be referable to the greater incidence of maternally acquired antibody to this virus as compared to passively acquired antibody to the WEE virus. An inquiry dealing with this possibility forms the basis of the present communication.

MATERIAL AND METHODS

The study was initiated in July 1953 and called for the collection of 1,000 paired maternal and infant bloods for purposes of antibody assay. The maternal blood specimens were to be obtained upon admission of the mother to the hospital, but prior to delivery, and infant bloods were to be obtained from the umbilical cord at delivery. The large number of individuals selected for the sample was based on earlier studies[8] on inapparent infections with WEE and SLE viruses in the Bakersfield, California area, which showed that about 10 per cent of the general population possessed antibodies to WEE virus and about 30 per cent possessed antibodies to SLE virus.

Collection of blood specimens. The collection of maternal and infant bloods was made a part of the routine procedure of the obstetrical service at the Kern County General Hospital, Bakersfield, California. Blood specimens were obtained by the staff of the obstetrical service from a total of 1,009 mothers and their infants; with the exceptions noted below, these were tested for complement-fixing and neutralizing antibodies to the WEE and SLE viruses. To study the disappearance of passively acquired antibody, blood specimens were subsequently obtained from many of the infants whose cord blood contained complement-fixing or neutralizing antibodies to either or both of these viruses.

Neutralization tests. Stock suspensions of WEE and SLE viruses were prepared by procedures described elsewhere.[10] Serial decimal dilutions of the 20 per cent stock virus were prepared in 10 per cent normal rabbit serum broth and to 0.15 ml amounts of the selected virus dilutions was added 0.15 ml of the serum to be tested. Known negative and positive sera were included in each test as controls. All sera were inactivated at 56°C for 30 minutes prior to use. The serum-virus mixtures were not incubated, but were injected into animals immediately after preparation. Each mixture was inoculated into groups of six mice.

All sera were first screened (undiluted) against 10 to 30 LD_{50} of virus as calculated by the Reed-Muench[13] method. In tests with the WEE virus, 4 to 5-week-old Swiss mice were inoculated intracerebrally with 0.03 ml amounts of the serum-virus mixtures, and in tests with the SLE virus, 2 to 3-day-old mice were inoculated intraperitoneally with 0.03 ml amounts of serum-virus mixtures Mortality ratios (number of mice that

* This study was supported in part by a grant (E-751) from the National Institute of Allergy and Infectious Diseases, United States Public Health Service, Department of Health, Education and Welfare.

TABLE 1

Age distribution of laboratory proved cases of Western equine and St. Louis encephalitis, California, 1953–1957

Age (yrs)	Western equine encephalitis		St. Louis encephalitis	
	No. cases	Per cent	No. cases	Per cent
Under 1	31	52.5	1	0.6
1–4	6	10.2	18	11.7
5–9	5	8.5	27	17.6
10–19	6	10.2	29	18.9
20–29	2	3.4	18	11.7
30–39	2	3.4	19	12.3
40–49	3	5.0	12	7.8
50–59	2	3.4	15	9.7
60+	2	3.4	14	9.1
Unknown	—	—	1	0.6
Total	59		154	

died/number of mice inoculated) were interpreted as follows: 0/6 and 1/6, positive; 2/6, inconclusive; and 3/6, 4/6, 5/6 and 6/6 negative.

Serum specimens which gave positive or inconclusive results were retested by the varying virus-constant serum method, in which undiluted serum is tested against several serial dilutions of virus. Serum-virus mixtures were prepared as indicated above, and inoculated intraperitoneally into infant mice (SLE virus) or intracerebrally into adult mice (WEE virus). Each test mixture was inoculated into a group of six mice. Neutralization indices, which represent the difference between the amount of virus neutralized in the presence of the test serum and the amount neutralized in the presence of normal human control serum, are presented as the reciprocal of the logarithm. Indices of 0.0 to 0.9 were considered to indicate absence of antibody and indices of 1.0 or greater to indicate the presence of antibody.

Complement fixation test. Complement fixation tests were performed essentially by the technique described in recent publications from this laboratory.[14, 18] The WEE antigen was derived from embryonated eggs,[11] and the SLE antigen was prepared from infant mouse brains by the acetone-ether extraction method of Casals.[2] All sera were inactivated at 60°C for 20 to 30 minutes before use. The starting serum dilution was 1:8 and all sera which fixed complement in this or higher dilutions were considered positive.

RESULTS

Incidence of WEE and SLE antibodies in maternal blood. Table 2 summarizes the results of the tests for the presence of WEE and SLE neutralizing and complement-fixing antibodies in the blood of the mothers forming the study group.

Of the 1,009 maternal bloods examined for complement-fixing antibody, eight, or 0.8 per cent, possessed demonstrable antibody to the WEE virus and 31, or 3.2 per cent, possessed demonstrable antibody to the SLE virus. The proportion of individuals with complement-fixing antibody to the SLE virus was thus four times larger than that with antibody to the WEE virus. Of the 1,008 mothers tested for neutralizing antibody to the WEE virus, 110, or 10.9 per cent, were positive, and of the 997 mothers tested for antibody to the SLE virus, 270, or 27.1 per cent, were positive. This gives a ratio of approximately 2.5 individuals with SLE-

TABLE 2

Proportion of mothers with antibodies to Western equine and St. Louis encephalitis viruses

Antibody	Total tested	Mothers tested		
		Number		
		With antibody*	Negative	Sera AC†
Complement-fixing				
WEE	1009	8 (0.8%)	993 (98.4%)	8 (0.8%)
SLE	1009	31 (3.2%)	969 (96.0%)	9 (0.9%)
Neutralizing				
WEE	1008‡	110 (10.9%)	898 (89.0%)	—
SLE	997§	270 (27.1%)	727 (72.9%)	—

* Antibody = complement-fixing titer ≥ 1:8; neutralization index ≥ 1.0.

† AC, serum anticomplementary.

‡ Insufficient serum for test on 1 individual.

§ Insufficient serum for test on 12 individuals.

TABLE 3

Relationship between length of residence in urban or rural areas of endemic region (Kern County, California) and incidence of WEE-neutralizing antibody in blood

Years of residence	Mothers tested		Distribution of mothers by residence*				
			Urban		Rural		Unknown
	Total	Number with antibody†	Total tested	Number with antibody	Total tested	Number with antibody	Number with antibody
0–4	335	26 (7.8%)	153	12 (7.8%)	182	14 (7.7%)	
5–9	279	25 (8.9%)	123	11 (8.9%)	156	14 (8.0%)	
10–14	176	21 (11.9%)	91	8 (8.8%)	84	12 (14.3%)	1
15–19	99	19 (19.2%)	63	9 (14.3%)	36	10 (27.8%)	
20–24	63	11 (17.5%)	41	6 (14.6%)	22	5 (22.7%)	
25–29	32	5 (15.6%)	24	2 (8.3%)	8	3 (37.5%)	
30+	24	3 (12.5%)	15	3 (20.0%)	9	0 0	
Total	1008	110 (10.9%)	510	51 (10.0%)	497	58 (11.7%)	1

* Category based on present address.

† Antibody = complement-fixing titer $\geq$ 1,8; neutralization index $\geq$ 1.0.

TABLE 4

Relationship between length of residence in urban or rural areas of endemic region (Kern County, California) and incidence of SLE-neutralizing antibody in blood

Years of residence	Mothers tested		Distribution of mothers by residence*				
			Urban		Rural		Unknown
	Total	Number with antibody†	Total tested	Number with antibody	Total tested	Number with antibody	Number
0–4	334	50 (14.9%)	153	23 (15.0%)	181	27 (14.9%)	
5–9	276	73 (26.4%)	122	22 (18.0%)	154	51 (33.1%)	
10–14	175	57 (32.6%)	90	27 (30.0%)	84	30 (35.7%)	1‡
15–19	95	35 (36.8%)	61	19 (31.1%)	34	16 (47.1%)	
20–24	61	28 (45.9%)	41	16 (39.0%)	20	12 (60.0%)	
25–29	33	17 (51.5%)	24	11 (45.8%)	8	6 (75.0%)	
30+	23	10 (43.5%)	15	5 (33.3%)	9	5 (55.6%)	
Total	997	270 (21.1%)	506	123 (24.3%)	490	147 (30.0%)	1‡

* Category based on present address.

† Antibody = complement-fixing titer $\geq$ 1,8; neutralization index $\geq$ 1.0.

‡ This individual possessed neither complement-fixing nor neutralizing antibody.

neutralizing antibody for each individual with WEE-neutralizing antibody. (In passing, it may be noted that 48 mothers had neutralizing antibodies to both viruses.)

That the proportion of individuals with complement-fixing antibody to the WEE or SLE viruses should be lower than the proportion of individuals with neutralizing antibody to the same agents was not unexpected. The presence of complement-fixing antibody is transitory, and hence generally accepted as evidence of recent infection, whereas neutralizing antibody, which usually persists for years following infection, is similarly accepted as evidence of experience with the particular antigen at some time in the recent or remote past. (None of the mothers in this study gave a history of recent illness.)

The marked differences between the proportion of mothers possessing demonstrable antibodies to WEE virus and those possessing antibody to the SLE virus may be due to one or more of several factors. One explanation for such a difference lies in the possibility that although both viruses are carried by the same mosquito vector, there may be a significant difference in their dissemination, the SLE virus being the more widely seeded throughout the population; another possibility, purely speculative at this time, is that the SLE virus may be transmitted by another vector or method. The difference might also be due to possible differences in the temporal persistence of antibody to the two viruses. Also, length of residence in an endemic area might have some bearing on the difference in proportion of individuals with antibodies to these two viruses.

Comparison of complement-fixing and neutralizing antibody levels in maternal blood. The number of individuals with complement-fixing antibody to either the WEE or the SLE virus was too small to afford significantly meaningful comparisons. There was some suggestion that the higher the neutralization index, the higher the complement-fixing antibody titer. However, as Table 2 shows, and as might be expected from a cross sectional study of a population, neutralizing antibody (often at a high level) was encountered in the absence of complement-fixing antibody. Complement-fixing antibody, however, was not encountered in the absence of neutralizing antibody.

Relationship of length of residence to acquisition of neutralizing antibody. Tables 3 and 4 present data relating to the length of residence in Kern County, an endemic area of Western equine and St. Louis encephalitis, and the presence of demonstrable neutralizing antibody in the blood.

With respect to WEE virus, Table 3 shows that of 335 mothers who had lived in Kern County for less than five years, 26 or 7.8 per cent had neutralizing antibodies. Of the 279 mothers who had lived in the county for five or more years but less than 10 years, 25 or 8.9 per cent had neutralizing antibodies. The proportion of mothers with neutralizing antibodies to WEE virus increased with years of residence, reaching a maximum of 19.2 per cent in the 15 to 19 year period. A similar relationship between the proportion of individuals with SLE-neutralizing antibody and length of residence in an endemic area is shown in Table 4. It will be noted, however, that the proportion of individuals in each residence group with neutralizing antibodies to the SLE virus is two to three times higher than the proportion with neutralizing antibodies to the WEE virus. Thus, as shown in Table 4, 51.5 per cent of the mothers in the 25 to 29 year residence group possessed neutralizing antibodies to SLE virus in contrast to only 15.6 per cent in the same group who possessed neutralizing antibody to the WEE virus.

When the data are analyzed with reference to urban and rural areas of residence, it is found that not only does the proportion of mothers with neutralizing antibodies for WEE or SLE viruses increase with years of residence within the endemic region, but also that the proportion of mothers with antibodies to these viruses is markedly higher in the group domiciled in rural areas.

It would thus appear, insofar as the present data go, that in an endemic area such as Kern County, the SLE virus is much more widely disseminated in the human population than is the WEE virus and that such dissemination is appreciably greater in rural areas where the opportunities for exposure to the mosquito

TABLE 5

Transplacental passage of WEE and SLE antibodies

Antibody	Total paired bloods tested	Number of maternal bloods		Number of cord bloods	
		With antibody*	Negative	With antibody*	Negative
Complement-fixing					
WEE	83†	6	77	8	75
SLE	84†	25	59	30	54
Neutralizing					
WEE	1004‡	108	896	109	895
SLE	973§	251	722	248	725

* Antibody = complement-fixing titer $\geq$ 1:8; neutralization index $\geq$ 1.0.

† Excludes anticomplementary sera, sera in insufficient amounts for test, and "not done." See text and also Table 6.

‡ Five pairs of sera in insufficient amount for test.

§ Thirty-six pairs of sera in insufficient amount for test.

TABLE 6

Western equine encephalitis virus. Distribution of complement-fixing antibody titers of maternal and cord bloods

Maternal bloods		Number of cord bloods with titer of				Specimens*		
Titer	No. of specimens	<1:8†	1:8	1:16	1:32	AC	QNS	ND
<1:8†....	993	75	2			40	4	872
1:8.....	2		1			1		
1:16.....	3			3				
1:32.....	3			1	1	1		
AC.....	8	2				1		5
Totals..	1009	77	3	4	1	43	4	877

* AC, anticomplementary; QNS, quantity insufficient for test; ND, not done (see text).
† <1:8 = negative.

TABLE 7

St. Louis encephalitis virus. Distribution of complement-fixing antibody titers of maternal and cord bloods

Maternal bloods		Number of cord bloods with titer of				Specimens*		
Titer	No. of specimens	<1:8†	1:8	1:16	1:32	AC	QNS	ND
<1:8†....	969	54	5			36	2	872
1:8.....	18		5	10		3		
1:16.....	8		1	4	2	1		
1:32.....	4				2	2		
1:64.....	1				1			
AC.....	9	2	1					6
Totals..	1009	56	12	14	5	42	2	878

* AC, anticomplementary; QNS, quantity insufficient for test; ND, not done (see text).
† <1:8 = negative.

vector (*Culex tarsalis*) are much greater than they are in urban areas.

Transplacental passage of antibodies. Not all of the 1,009 *pairs* of blood specimens from mothers and their infants were examined for antibody content. In some instances, the matching pairs were not examined because one or the other of the two specimens was anticomplementary or its quantity was insufficient for test purposes. In the great preponderance of instances, however, maternal and infant bloods were not tested as a pair for complement-fixing antibodies since prior screening of the mother's blood had revealed no demonstrable antibody, and it was assumed that the cord blood would therefore also be negative. Thus, complement-fixing antibody determinations were not done on the bloods of 872 infants of the 993 mothers whose blood possessed no demonstrable complement-fixing antibody (*cf.* Table 6), nor were tests done on the bloods of 872 infants of the 969 mothers whose sera contained no demonstrable complement-fixing antibody to the SLE virus (*cf.* Table 7).

Screening of all the maternal bloods by the complement fixation test showed that only eight possessed complement-fixing antibody to the WEE virus and 31 possessed such antibody to the SLE virus. These positive bloods were then paired with the corresponding infant bloods and re-examined together with a number of similarly paired bloods from negative mothers. The results are presented in Table 5; this table does not, it should be pointed out, include two mothers with WEE antibodies, and six mothers with SLE antibodies, since the cord bloods of their infants were anticomplementary. All six infants whose mothers possessed complement-fixing antibody to WEE virus also had such

TABLE 8

Western equine encephalitis virus. Distribution of neutralizing antibody titers of maternal and cord bloods

Maternal blood		Number of cord bloods with neutralization index of						QNS*
Neutralization index	No. of specimens	0.0-0.9	1.0-1.4	1.5-1.9	2.0-2.4	2.5-2.9	3.0-3.4	
0.0-0.9†....	898	895	1					2
1.0-1.4.....	4		2	2				
1.5-1.9.....	28		1	14	11	1		1
2.0-2.4.....	45		1	8	21	12	2	1
2.5-2.9.....	27				15	9	3	
3.0-3.4.....	6				3	2	1	
QNS.....	1			1				
Total.....	1009	895	5	25	50	24	6	4

* QNS, quantity insufficient for test.
† This neutralization range not significant, *i.e.*, negative.

antibody; similarly, all 25 infants whose mothers' blood contained SLE antibody also had such antibody. It will be noted from Tables 5 to 7 that two infants apparently possessed WEE complement-fixing antibody at birth, although their mothers' blood was negative and five infants similarly possessed SLE complement-fixing antibody although the maternal blood was negative. These discrepancies are reasonably attributable to the subjective error inherent in reading the results of complement fixation tests. Thus, as is shown in Table 6 and 7, the two positive WEE and five positive SLE cord bloods had titer readings of only 1:8; since a variation of one dilution step in serum end-point titer is generally accepted as within the experimental error of the method in replicate testing,[7] it appears possible that on replicate testing the infant cord bloods might have given titers of <1:8, *i.e.*, negative; or similarly, retesting of the maternal bloods might have given fixation interpreted as positive as 1:8.

With respect to neutralizing antibody, Table 5 shows that of the 1,004 pairs of mother-infant bloods examined for WEE antibody, 108 mothers and 109 infants were positive. Of the 973 pairs of mother-infant bloods tested for SLE antibody, 251 mothers and 248 infants were found to be positive. Maternal and infant bloods were always tested as pairs. The several discrepancies seem explicable as experimental errors. Thus, the infant who apparently possessed WEE-virus neutralizing antibody although his mother was negative, had an index of 1.0 to 1.4, or just above the line (0.0 to 0.9) set for "negative" results (*cf.* Table 8). Similarly, two of the three infants whose cord bloods were negative for SLE-neutralizing antibody were progeny of mothers with the minimal neutralization index (1.0 to 1.4) considered significant (*cf.* Table 9). The neutralization index of the third mother was in the 1.5 to 1.9 range. Aside from these four discrepancies, which may be considered as within the norm of laboratory variation, the agreement in findings on the maternal and respective infant bloods was excellent. This is illustrated in Tables 6 to 9, which compare the antibody levels of the paired maternal and infant bloods. In the entire study series, there were only eight mothers with complement-fixing antibody to the WEE virus; despite the numerical paucity of positives, the agreement between the antibody titers obtained on the blood of the mother and on the blood of her infant was good (*cf.* Table 6). The complement-fixing antibody levels to the SLE virus of the 31 mothers and their infants were similarly in very good agreement (*cf.* Table 7).

TABLE 9

St. Louis encephalitis virus. Distribution of neutralizing antibody titers of maternal and cord bloods

Maternal blood		Number of cord bloods with neutralization index of						
Neutralization index	No. of specimens	0.0–0.9	1.0–1.4	1.5–1.9	2.0–2.4	2.5–2.9	3.0–3.4	QNS*
0.0–0.9†	727	722						5
1.0–1.4	28	2	8	10	2	1	1	4
1.5–1.9	43	1	3	16	10	9	2	2
2.0–2.4	61		1	9	31	13	3	4
2.5–2.9	38			2	10	15	11	
3.0–3.4	100		1		1	11	78	9
QNS	12				4	2	3	3
Total	1009	725	13	37	58	51	98	27

* QNS, quantity insufficient for test.

† This neutralization range not significant, *i.e.*, negative.

Comparison of the maternal and infant neutralizing-antibody titers to the WEE and SLE viruses is presented in Tables 8 and 9, respectively. It will be seen that although the correlation is not as good as in the case of complement-fixing antibody, it is nevertheless noteworthy. Although there is some spread in the neutralization indices of mother and child, the existence of a correlation between the maternal and infant antibody levels is readily discernible in Tables 8 and 9.

Disappearance of placentally transmitted antibody. One hundred and twenty-four infants whose cord blood at birth contained neutralizing antibody were subsequently rebled at some time over the succeeding 13 months. Data on the rate of disappearance of antibody are presented in Figures 1 and 2. In these figures, each bar represents one individual; the top of the bar represents the antibody titer at birth, and the bottom of the bar represents the antibody titer at the time of the second bleeding, the intervals between the two bleedings being indicated at the bottom of the chart.

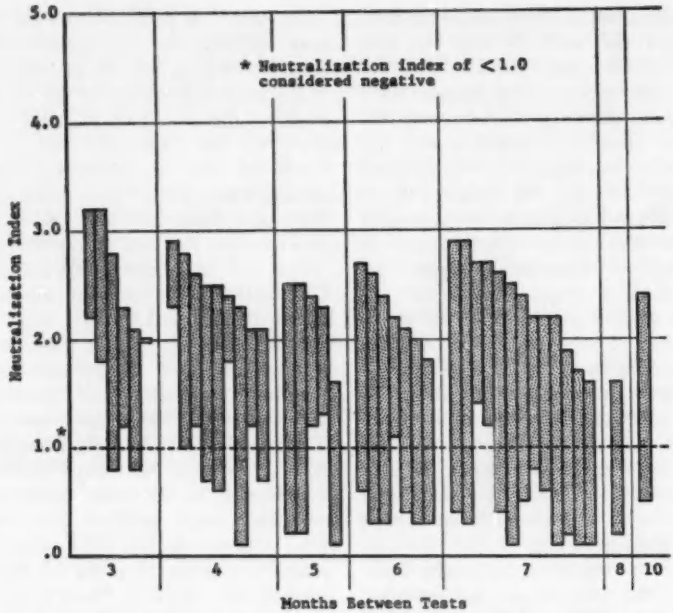

FIG. 1. Loss of placentally transmitted neutralizing antibody to Western equine encephalitis virus.

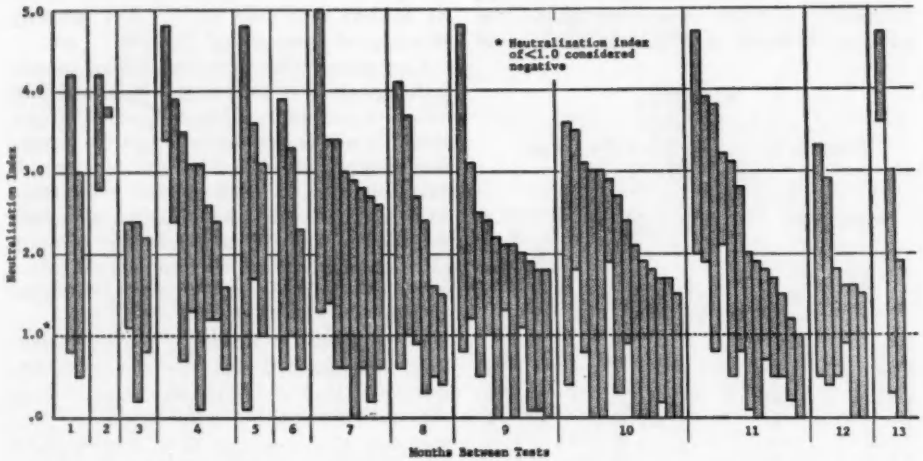

FIG. 2. Loss of placentally transmitted neutralizing antibody to St. Louis encephalitis virus.

Figure 1, which shows the rate of disappearance of antibody to the WEE virus, is based on findings obtained in 42 infants, each bled once at some interval between three and ten months after birth. Although the number of individuals studied is small because of various difficulties associated with the collection of the second blood specimens (lack of parental cooperation, inability to locate families, etc.) the trend toward rapid loss of the passively-acquired antibody is

evident. Thus, 30 per cent of the infants bled at three months had lost their antibody and at four months 45 per cent had lost their antibody, the proportion increased until, of those infants bled at seven months, 85 per cent no longer possessed antibody. A rapid loss in neutralizing antibody to the SLE virus was also observed and the data derived from 82 infants that were studied are presented in Figure 2. As was noted with the WEE-neutralizing antibody, some individuals lost their antibody to the SLE virus quite early, others somewhat later; the proportion of those who lost antibody increased with time, so that of those bled at six months, from 75 to 85 per cent were without antibody, and by 12 months virtually all children had lost the passively acquired antibody.

DISCUSSION

Clinically and epidemiologically, Western equine encephalitis differs from St. Louis encephalitis in the propensity of the former to attack very young individuals, especially infants. There is no unequivocal explanation for this difference in age selectivity, but it was considered possible that differences in maternal transmission of immune bodies might be responsible. The finding that approximately 11 per cent of the mothers studied possessed neutralizing antibody to the WEE virus as compared to 27 per cent who possessed neutralizing antibody to the SLE virus is in agreement with previous surveys[5] on the presence of antibody in the general population of the endemic area (Bakersfield, California) where the present studies were done; it would therefore appear that although both viruses are transmitted by the same arthropod vector (*C. tarsalis*), the SLE virus for some as yet unknown reason is appreciably more widely disseminated than is the WEE virus. If we accept that transplacental passage of antibody to both viruses occurs with equal efficiency, the difference in the proportion of mothers possessing WEE antibody and those possessing SLE antibody, *viz.*, 11 per cent versus 27 per cent, does not appear to be of sufficient magnitude to explain the rarity of St. Louis encephalitis in infants under one year of age and the high attack rate of Western equine encephalitis for this same age group.[12]

Studies on the placental passage of antibodies (complement-fixing and neutralizing) to the WEE and SLE viruses showed that within the limits of experimental error the antibody content of the infant's blood was the same as that of the mother's blood. As is true of a number of other viral antibodies studied (*e.g.*, mumps,[3] Japanese B encephalitis[4] and poliomyelitis[1]) antibodies to the WEE and SLE viruses traverse the placental barrier without difficulty. No indisputable evidence was obtained to indicate that SLE-neutralizing antibody acquired from the mother persists for significantly longer periods than does neutralizing antibody to the WEE virus, and hence the relative freedom of the infant age group from St. Louis encephalitis cannot be ascribed to a difference in temporal persistence of antibody. Insofar as the present data go, they indicate that neutralizing antibody to the WEE and SLE viruses disappeared at about the same rate and at essentially the same rate as that reported for antibody to other viral agents.[1, 3, 4]

SUMMARY

Examinations for the presence of neutralizing antibody to the Western equine encephalitis (WEE) and St. Louis encephalitis (SLE) viruses were made of blood specimens from parturient mothers residing in an endemic area of Western equine and St. Louis encephalitis. It was found that 11 per cent of the mothers studied possessed neutralizing antibody to the WEE virus and 27 per cent possessed neutralizing antibody to the SLE virus; there also was evidence of a direct relationship between the length of residence in the area and the acquisition of such antibody.

It was found that both complement-fixing and neutralizing antibodies to these two viruses readily pass the placenta, but no evidence was obtained that the difference between the proportion of infants possessing antibody to the WEE virus and those possessing antibody to the SLE virus is responsible for the remarkable difference in the attack rates of these two agents for infants. The temporal persistence of passively acquired neutralizing antibody to the WEE and SLE viruses appeared to be no greater than that reported for antibodies to other viral agents.

ACKNOWLEDGMENT

The collection of the blood specimens on which this study is based would not have been possible without the valuable cooperation of

Dr. Howard Grove, Chief, and Vera Jolliff, R.N., Head Nurse, Obstetrical Staff, Kern County General Hospital, and the assistance of Drs. Burnett Cole, David Jamieson, Thomas Cummings, Kenneth Turner and Bernard Arellano of the resident staff. Study material was also collected through the active interest and assistance of Myona Morrison, P.H.N., Ann Farrell, P.H.N. and Wennona Nabers, R.N., of the Kern County Health Department and of Miss Harriet Hanson, medical student.

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AN ATTEMPT TO ERADICATE MALARIA BY THE WEEKLY ADMINISTRATION OF PYRIMETHAMINE IN AREAS OF OUT-OF-DOORS TRANSMISSION IN VENEZUELA

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Anophelines are senior to man on the earth. The pursuit of man by adult anophelines thus appears to be an acquired character in the evolution of a species. Therefore, anophelines, from the standpoint of their behavior towards man, may be classified as follows: (a) outdoor biters and outdoor resters; (b) indoor biters and outdoor resters; (c) outdoor biters and indoor resters; and (d) indoor biters and indoor resters. When mosquitoes are indoor resters, they may be killed by indoor spraying with residual insecticides, malaria transmission being interrupted by shortening the life span of the mosquitoes. In this case, malaria is responsive to the insecticide and eradication may be obtained with insecticides alone. On the other hand, malaria may be refractory to the insecticide when the vectors are outdoor resters. Under conditions where transmission is due to both indoor biters and resters and to outdoor biters and resters, natural partial refractoriness results when indoor transmission has been eliminated by the use of residual insecticides. Eradication of partially refractory malaria can, therefore, only be obtained by other antianopheline measures or by antiparasite measures.

Eradication, exclusion, or reduction of the vector are generally unsuitable for wide areas under tropical conditions. The hope of obtaining eradication of partially refractory malaria under Venezuelan conditions depends, therefore, on antiparasite measures.

Among the modern antimalarials, only primaquine is able to produce radical cure of *vivax* infection but because of its inherent toxicity, it should not be administered to a large number of persons by nonmedical personnel. Weekly treatment with chloroquine may interrupt transmission for a time sufficient to bring about the natural death of the infection. The drug is generally well tolerated but has some disadvantages in wide field application. It is bitter and hence difficult to administer to children; it may produce stomach aches and vomiting; and finally, an occasional unexpected death in small children may occur.

Pyrimethamine is also well tolerated, does not produce side effects, is tasteless and according to Coatney *et al.*,[1] weekly administration of 25 mg for 8 or more weeks results in suppressive cure of many *Plasmodium vivax* infections in adults. In line with these facts it was decided that the single weekly dose for adults would be 50 mg and that the administration would be continued for 24 weeks in the hope of obtaining radical cure of all infections and thereby solve the problem of partially refractory malaria in two areas of Venezuela.

THE PROBLEM

When malaria is responsive to a particular insecticide, its reduction is by crisis, the disease manifesting a rapid diminution in its annual rate of prevalence, the effect being the consequence of a virtually complete interruption of transmission. When malaria is partially refractory to the insecticide, its reduction is by lysis, a gradual decline of incidence, as a result of partial interruption of transmission. When an analysis was made at the beginning of 1951 of the progress of the malaria eradication program in Venezuela, it was found that two areas showed partially refractory malaria.[3, 5]

In Eastern Venezuela there is a small area where *Anopheles aquasalis* with eggs of the *emilianus* type is the responsible vector. It belongs to a special group, deme III of Senior-White,[6]

* The authors wish to thank Dr. Arturo Luis Berti, Chief of the Division, for the facilities and help given while the work was being carried out.

which, because it bites and rests out-of-doors, has been able to maintain a low grade transmission in spite of 2.3 sprayings per year (average) since 1950. In this area *Anopheles darlingi* and *Anopheles nunez-tovari* are absent, but *Anopheles albimanus* is present in small numbers.

In Western Venezuela is found another area which has continued to exhibit new infections in spite of an average of 2.0 sprayings per year since 1950. The predominant vector is *A. nunez-tovari*, but *A. darlingi* exists in certain areas. *A. nunez-tovari* is a delicate mosquito in comparison to *A. aquasalis*. In spite of the fact that it produced malaria of high endemicity with some spleen indices in the area close to 100%, the reduction obtained with residual sprayings was more pronounced than in the *A. aquasalis* area. In areas with pastures or annual crops of small plants, transmission was interrupted by the insecticide. In areas where the houses were not close to the jungle, malaria disappeared when the inhabitants were instructed to take weekly suppressive doses of chloroquine supplied to each house by the rural visitor, or more if they felt sick. This method of administering chloroquine was not effective in the districts where the houses were near the forest or in banana plantations, where the particular ecological conditions allow the anophelines to live long enough for the parasite to reach the sporozoite stage. Chloroquine failed to stop transmission under these conditions in spite of being widely used by the inhabitants of the area. The tablets were taken by the people and not sold because they had no value due to the fact that they were supplied free to all.

Also, in part of the *aquasalis* area chloroquine failed to stop transmission when given as a single dose monthly from 1952 to 1956. In both areas, now that *P. vivax* is the only parasite present, a number of people have shown parasites, as new infections or relapses, without clinical symptoms. Since these people do not feel sick, they take no medication with the result that mosquitoes feeding on them become infected. Children may become infectors because the mothers are reluctant to give them the bitter drug.

MATERIAL AND METHODS

Localities in Eastern and Western areas which showed malaria parasites in 1956 and in the first half of 1957 were selected for the weekly suppressive treatment with pyrimethamine. Some nearby localities were also included although parasites were not present at the time. In the Eastern area there were 174 localities with 3,084 houses and 16,416 inhabitants, that is, an average of 17.7 houses per locality. In the Western area there were 735 localities and 17,638 houses and 95,579 inhabitants, an average of 24.0 houses per locality. This small number of houses per locality is a clear sign of the dispersion of the population in the rural areas where the work was carried out. The total number of inhabitants treated was 111,995.

Most of the localities were hard to reach. Housing was poor, mostly with thatched roofs and mud walls. All houses had been numbered by the spraying squads. An envelope with two cards, to be signed each time the rural visitor made his round, was placed in a prominent place on the inside wall of each house. One card (red) was for the drug dispensers and the other (green) for the slide collectors.

Rural visitors in charge of drug distribution made weekly rounds, covering 80 to 150 houses, according to difficulties of transportation and distances between houses. There were 168 of these workers. Rural visitors whose job was to collect slides made monthly rounds. Each of these workers territory covered that of approximately four drug dispensers. There were 127 of these rural visitors. Both types of rural visitors traveled mostly on horseback but some used boats with outboard motors and others used bicycles depending on the places to be visited. Each visitor had a fixed itinerary. There were 13 inspectors and 26 subinspectors to supervise the work.

The treatment sheets, one per house, with the names and ages of the inhabitants and 12 spaces for entering the doses given to each person, were carried in a folder in the order in which the houses were to be visited. Rural visitors in charge of drug distribution carried only pyrimethamine. They did not take slides even though they might encounter patients with fever. Visitors in charge of collecting slides were supplied with chloroquine to be given only to patients with fever. They made slides from persons with fever or recent history of fever and from those who appeared ill, or even from healthy persons, particularly children, in order to fill their monthly quotas.

When a drug dispenser got to a house, he

gave each inhabitant the drug, assured himself that it was swallowed, and checked each name on his treatment sheet. If some person was absent, tablets were left for him, but that treatment was not entered in the record because it was not taken in the presence of the visitor. It is considered probable that most of the treatments left were actually taken, in which case more treatments were taken than actually recorded. The visitor always tried to find those persons who had not taken the drug and when located were given the drug on the spot. Temporary visitors in a locality were given the drug and their names recorded on a special sheet.

Pyrimethamine was given according to the following regimen: 2 tablets of 25 mg to persons of 15 years or over, one tablet of 25 mg to children between 5 and 14 years, and half a tablet, or 12.5 mg to children below 5. Every person was given treatment except infants less than one-month old. Babies below one year received their half tablet in a suspension made in chlorinated water and given generally by their own mothers.

The cooperation of the people was excellent. Occasionally persons refused the drug at the start of the campaign, but after talking with the doctor, they almost invariably entered the study and continued in good spirit. To stimulate interest, all those who took the full 24 weekly treatments were given a lottery ticket for prizes consisting of sewing machines, bicycles, etc. As an incentive for the children, each was given sugar candy after the tablet was swallowed.

Drug dispensers received a bonus of about $20 (U.S.) for each month in which no parasites were found in the blood of the people of their sector. Slide collectors, on the other hand, received about $25 (U.S.) as a bonus each month in which parasites were found in one or more persons of their sector. Each visitor tried to do his best in order to qualify for the bonus.

When the work was well underway, Dr. G. Robert Coatney of the National Institutes of Health was invited to visit the project in order that he might appraise the efficiency of the drug administration and other procedures. Our thanks are extended for this cooperation.

Organization of the work in the field was carried out during April, May and June (1957). Some sectors were too large and some were too small and while this was being adjusted, chloroquine was the only drug administered. We knew that administration would be irregular in some areas due to the above conditions and pyrimethamine was withheld in order not to encourage the development of resistance. By the end of June, sector organization was complete and the switch was made to pyrimethamine the first Monday in July and continued to the 21st of December. In early December all chloroquine left in the houses of the Western area was collected. Householders were then without drug for self medication.

Beginning in January (1958) house visits were carried out every two weeks in the treated areas. Rural visitors were instructed to collect blood smears only from persons suspected of being infected. These people received one single dose of chloroquine at the time the slide was taken. Persons found infected were given primaquine, 15 mg daily for 14 days in order to bring about radical cure.

Outside the active search for infected persons by the rural visitors, a thorough passive search was made at each of the 54 medical dispensaries in the area. At these installations the nurses were requested to make a slide from each person who came to the dispensary and was suspected of being infected with malaria. If a nurse made 25 or more slides per month, she received a bonus of $15 (U.S.) and an additional $2 for each slide found positive. The "incentive pay" arrangement worked well.

RESULTS

When pyrimethamine administration was begun in 1957, malaria had already decreased in the localities under test. As stated above, this area had been sprayed regularly since 1950, and some parts since 1948. As shown in Table 1,

TABLE 1

Morbidity indices (positive slides per 100,000 house visits) in years before pyrimethamine suppressive treatments

Year	Eastern Area (174 localities)	Western Area (735 localities)
1945–1950	2595	3273
1951	3271	2150
1952	5226	1452
1953	1345	1670
1954	2119	619
1955	1248	295
1956	1104	360

the decrease in the morbidity indices was slow, with small increases during some years. The decrease in the Eastern Area was slower than in the Western Area, in spite of the fact that the degree of endemicity was less, as shown in Table 2. The most pronounced decrease was observed in the Western Area. This might have been due to the fact that chloroquine was more widely used in this area. The inhabitants of each house had been supplied with tablets since 1954. Likewise chloroquine probably contributed to the reduction of the spleen indices observed in the Western Area (Table 2). At the same time, since *A. aquasalis* is a more active vector than *A. nunez-tovari* in sprayed localities, the differences observed in the two areas may be due to the vectors.

TABLE 2

Spleen indices of some representative localities in both treatment areas

Localities	Highest spleen index		Lowest spleen index	
	Index	Year	Index	Year
Eastern Area				
Nurucual, Sucre	65.5	1942	26.3	1955
Santa Fe, Sucre	55.4	1942	14.0	1955
Western Area*				
Abejales, Tachira	92.5	1948	6.6	1956
Boca Grita, Tachira	95.2	1948	5.4	1955
La Fria, Tachira	100.0	1948	7.7	1955
Navay, Tachira	94.1	1948	4.1	1956
Palmarito, Merida	86.5	1947	7.7	1956
Pto. Nuevo, Tachira	100.0	1949	6.4	1955

* Localities with only *A. nunez-tovari* (*A. darlingi* absent).

In Table 3 the parasite formula is presented for the municipios in the pyrimethamine-treated areas. The basic data for 1941–1945 show the difference in the degree of endemicity between the Eastern and Western Areas. It will be noted that *Plasmodium falciparum* and mixed infections were much higher in the Western Area and that the decrease of *P. falciparum* after the start of spraying in 1947 or 1948 was slow in both areas. In areas of responsive malaria its disappearance is sudden.[4] The slow decrease of this species is characteristic of the reduction of malaria by lysis. Again, referring to Table 3, it will be seen that by 1950 *P. falciparum* had reached a low figure. This decrease was due solely to the use of DDT as an indoor residual spray which, for all practical purposes, eliminated inside-the-house transmission. (No drug was administered until 1952 when chloroquine was used in small quantities.) The remaining outdoor-rester anophelines do not have a life span long enough to transmit *P. falciparum* although sufficient for the transmission of *P. vivax*. Furthermore, *P. falciparum* infections

TABLE 3

Parasite formula for all the municipios in which there were pyrimethamine-treated areas

Year	Eastern Area*					Western Area*				
	Number of infections	V	F	M	Mixed	Number of infections	V	F	M	Mixed
1941–1945	8313	43.0	49.4	4.1	3.4	1162	26.2	58.1	9.6	6.2
1946	856	55.1	41.1	1.0	2.7	551	38.1	55.2	1.4	5.3
1947	313	56.9	38.6	1.9	2.6	353	59.5	36.8	1.4	2.3
1948	160	55.0	38.1	3.8	3.1	192	62.0	32.8	1.6	3.6
1949	93	71.0	23.6	4.3	1.1	325	69.8	24.9	1.8	3.4
1950	73	93.2	4.1	0	2.7	1174	94.0	4.7	0.7	0.6
1951	423	85.1	13.2	0.5	1.2	1103	93.0	5.8	0.6	0.5
1952	780	88.8	10.3	0.4	0.5	1103	89.8	9.3	0.4	0.4
1953	229	92.1	7.0	0.9	0	1219	97.1	2.6	0	0.2
1954	219	96.3	1.4	2.3	0	644	99.7	0.2	0	0.2
1955	197	99.0	0.5	0.5	0	380	98.9	0.8	0	0.3
1956	262	100.0	0	0	0	491	99.6	0.4	0	0
1957	162	100.0	0	0	0	217	100.0	0	0	0

* V, vivax; F, falciparum; M, malariae.

TABLE 4

Monthly morbidity rates (P. vivax) *per 100,000 inhabitants and their index numbers*

Month	Normal for 5 localities (1938–1945)*		Pyrimethamine localities (1958)	
	Rates	Indexes	Rates	Indexes
January	1744	100.0	202	100
February	1530	87.7	718	355
March	1193	68.4	888	440
April	892	51.1	430	213
May	1261	72.3	652	323
June	1948	111.7	360	178
July	2296	131.6	494	245
August	3013	172.8	292	145

* Taken from Gabaldon.[1]

TABLE 5

Number of positive cases in the areas where weekly suppressive pyrimethamine treatments were carried out from July 1 to December 21, 1957

1957	Eastern Area	Western Area	Both areas
January to June	112	161	273
July to September	16	29	45
October	0	0	0
November	0	0	0
December	0	0	0

are more invasive, which calls for wider use of drugs, which, in turn, limits the length of the parasitemia over that of infection with *P. vivax* giving less opportunity for transmission.

P. vivax is now practically the only parasite present. It seems, however, that the present strains are different from the ones previously encountered in Venezuela. Prior to 1950 most *vivax* infections produced few relapses so that each case was considered a new infection; relapses were not considered in order to avoid mistakes in connection with malaria eradication. As shown in Table 4, *P. vivax* infections normally diminished during the dry season from January to April when transmission was reduced due to the decrease in the number of vectors. In the almost complete absence of transmission the number of infections should be further reduced. During 1958, however, the number of infections increased after January, pointing toward a strain of *P. vivax* with a marked tendency for relapse. What probably happened was that the strains of *P. vivax* producing few relapses disappeared along with *P. falciparum*. The relapse-producing strains of *P. vivax* which had more days of infectiousness for the mosquito, became the prevalent ones. This change in the life pattern of *vivax* malaria has increased the difficulties of malaria eradication in Venezuela.

With the beginning of weekly suppressive treatment with pyrimethamine, the number of cases began to decline and during the last 3 months of the study no malaria parasites could be found although a thorough search was made for them (Table 5). Transmission was interrupted with the disappearance of parasitemia and the first objective was realized.

The second objective, that of radical cure of all infections, however, was not achieved. In January 1958, relapses began to appear (Table 6), and increased progressively to a peak during the 9th to 12th weeks after the last suppressive treatment and then decreased through the 40th week.

As shown in Table 6, not all persons received one treatment per week during the 24 weeks of the test and some received less than 18 treatments. The number of relapses found in persons with less than 18 treatments, however, was less than the number found in persons who received 19 or more treatments. Most of the people received more than 19 treatments; the actual figures, however, are not available because of lack of mechanical tabulation of the data. Re-

TABLE 6

Infections found according to number of treatments received and weeks after last treatment

Weeks after last treatment	Persons who received 18 treatments or less	Persons who received 19 treatments or more	Total
1–4	2	4	6
5–8	10	19	29
9–12	14	52	66
13–16	10	23	33
17–20	8	19	27
21–24	6	24	30
25–28	9	3	12
29–32	9	3	12
33–36	2	0	2
37–40	1	0	1
Total	71	147	218

TABLE 7

Infections found according to age of persons and number of treatments received

Areas	Number of treatments received				Total
	1–6	7–12	13–18	19–24	
Eastern					
−1 year	2	0	0	0	2
1–4 years	1	0	0	7	8
5–14 years	1	2	4	20	27
15+ years	3	1	3	28	35
Total	7	3	7	55	72
Western					
−1 year	0	0	0	0	0
1–4 years	1	1	3	14	19
5–14 years	3	2	4	38	47
15+ years	6	16	18	40	80
Total	10	19	25	92	146
Both areas	17	22	32	147	218

TABLE 8

Variation in relapse rate according to age groups

Age (yrs)	Number of cases found			Specific age morbidity rates per 100,000		
	1956	1958	Index numbers 58/56	1956	1958	Index numbers 58/56
−1	32	2	6.2	770.0	70.9	9.2
1–4	195	27	13.8	1679.3	342.5	20.4
5–14	199	74	37.2	1077.2	582.0	54.0
15+	243	115	47.2	503.8	354.2	70.3
Total	669	218	32.6	811.2	389.3	48.0

lapses in persons with less than 18 treatments appeared to show the same trend as those who received 19 or more treatments.

Most of the relapses were observed in the groups of 5 to 14 years and of 15 years or more. Also in these age groups were more persons who took less than 18 treatments. This was due to the greater mobility of people of these ages and the consequent difficulty of the drug dispensers finding them in their homes. The two babies found positive in the Eastern Area were born in October 1957 and came down with *P. vivax* in March 1958. These cases were primary infections and not relapses. These infections were in different localities of the Eastern Area and relapses had been found in February; infections in the infants appeared more than 30 days later. It is evident, therefore, that not all infections found in 1958 were relapses, but due to the fact that the number of infections decreased in May and June when the rate of transmission normally increases, it would appear that most infections were actually relapses.

Although the number of infections found in the first half of 1958 (Table 7) was less in the Eastern Area than in the Western, the rates were higher in the former than in the latter. There were no imported cases in the Eastern Area but, as stated above, there were some new infections. The morbidity rate for this area was 877 per 100,000 in the first half of 1958. In the Western Area with several imported cases and apparently no new infections, the morbidity rate was 306 per 100,000 for the same period. These data show that it is much harder to interrupt transmission in the presence of *A. aquasalis* than in the presence of *A. nunez-tovari*.

An interesting fact in the prevalence of relapses in 1958 is the change of incidence according to age as shown in Table 8. In Venezuela the age group 1 to 4 years normally has the highest prevalence (Gabaldon, 1949). This prevalence was found in the areas where pyrimethamine was administered in 1956. In 1958, however, the age group with the most malaria was that between 5 and 14 years. Furthermore, the morbidity rates in those of 15 years or more in 1958 was not very different from that of the group 1 to 4 years old; also, a departure from the normal.

DISCUSSION

The data presented in this paper clearly show that in areas of partially refractory malaria it is impossible to eradicate the disease with insecticides alone. Eradication will, therefore, be possible only with the help of drugs. Chloroquinized salt may be of help in areas where salt distribution can be adequately controlled. In the two areas under consideration it was not possible to obtain such control and also it would have been too expensive because the people and the cattle use the same type of salt.

Weekly suppressive treatment with pyrimethamine was found effective in eliminating para-

sites from the blood and in stopping transmission. This measure, however, did not produce radical cure of vivax infections, indicating that if eradication is to be obtained by this method administration of the drug will have to be continued for a longer period, possibly up to two years, in order to allow for the natural death of the exoerythrocytic stages responsible for relapse. This is an expensive procedure, however, as the administration of drug with adequate parasitological supervision costs 11 to 21 cents per person per visit depending on the distance between houses.

With drugs now available and with conditions similar to the ones encountered here, there appears to be no alternative but to use pyrimethamine. Nevertheless, it is expected that the same doses, *i.e.*, 50 mg per adult, given every two weeks will be sufficient to prevent transmission. This regimen was adopted in July 1958. So far results have proved entirely satisfactory with no evidence of drug resistance.

Primaquine is a truly curative drug and can be used in that capacity if it is desired to cure all parasite-positive cases. During the first half of 1958 all infections were treated with primaquine for 14 days. This treatment is expensive. Because of the need for close medical supervision, patients were transported to treatment centers where they were housed and fed. Employed persons continued to receive their usual salaries with the result that our total costs amounted to $35 to $60 per patient. It is expected that with prolonged administration of pyrimethamine there will be little need for primaquine because of the absence of parasites from the peripheral circulation. Imported cases, however, will be treated with primaquine in order to insure eradication.

SUMMARY

Pyrimethamine was used in two areas of Venezuela with partially refractory malaria where the vectors were *Anopheles aquasalis* and *Anopheles nunez-tovari*.

During the months of July through December, 1957, a total of 111,995 people received pyrimethamine once weekly at a dose of 50 mg to adults and correspondingly smaller doses to children. The above dosages were eminently effective in stopping transmission as evidenced by the fact that no parasite-positive cases were found during the last three months of the project. Relapses appeared in both areas, however, after drug administration was stopped which showed that pyrimethamine as employed here did not produce suppressive cure of all vivax infections. It is hoped that pyrimethamine given at the same dosage and continued for a greater length of time in areas of partially refractory malaria will interrupt transmission until the infections die out.

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HEPATO-SPLENIC SCHISTOSOMIASIS IN MICE

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The clinical manifestations of *Schistosoma mansoni* infections in humans depends mainly on the degree to which 2 different organ systems are involved. Based on this dichotomy, Pons[12] recognized 2 more or less distinct syndromes which he designated intestinal and hepato-splenic schistosomiasis. The former is more frequently seen but the latter is by far the more serious. The onset of the hepato-splenic form of the disease is insidious and progresses over a period of years before reaching a critical stage. In advanced cases, the disease is characterized by hepatomegaly, splenomegaly, esophageal varices, ascites and anemia. Massive hemorrhage from ruptured esophageal varices is the most frequent specific cause of death.

Although the hepato-splenic form of schistosomiasis has received considerable attention, the pathogenesis of the disease remains in doubt. Its etiology has been related to (a) localized granulomatous lesions in the liver induced by the schistosome eggs, (b) portal cirrhosis provoked by toxin(s) produced by the adult worms, (c) the presence of dead worms in the liver, and (d) malnutrition. (For discussion and review of the literature on the etiology of hepato-splenic schistosomiasis see references 1, 5–7.) Each of the above listed pathologic factors could conceivably cause liver damage in a person infected with *Schistosoma mansoni*. However, since the complete hepato-splenic syndrome has not been knowingly produced in experimental animals, and thus has not been subject to controlled study, it has been impossible to determine the significance of these factors in the production of the hepato-splenic syndrome.

Warren and DeWitt[16, 17] have shown recently that portal hypertension and esophageal varices, both integral components of the hepato-splenic syndrome, develop in mice experimentally infected with *S. mansoni*. The purpose of this report is to characterize the hepato-splenic syndrome in mice and compare it with the human disease.

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MATERIALS AND METHODS

Young, adult, Swiss strain, albino mice, maintained on conventional pellet diet, were exposed individually to approximately 125 cercariae of a Puerto Rican strain of *S. mansoni* in 3 lots, according to the method of Olivier and Stirewalt.[11] Groups of mice were studied at 2 week intervals from the sixth to the 16th week after infection. Portal blood pressure was measured in mice under Nembutal anesthesia by introducing a 22-gauge needle connected to a strain gauge, into the portal vein. A yellow latex medium was injected into the portal system at a uniform pressure of 200 mm Hg to make special preparations for anatomic and photographic study.

Bromsulfalein retention tests were made using a modification of the procedure of Reinhold and Hutchinson.[13] The dye (phenol tetrabromosulfonphthalein sulfonate sodium) was prepared for injection by diluting a commercial stock solution containing 50 mg per ml with sterile physiologic saline to a concentration of 4 mg per ml. The final solution (0.3 ml) was injected into the tail vein of the test animals and 15 minutes later the mice were killed and blood samples were collected in capillary tubes from the incised portal vein. After the blood clotted, the serum was separated by centrifugation. Using accurately calibrated micropipettes, 0.02 ml of serum, 0.02 ml of 0.5N NaOH, and 0.2 ml of physiologic saline solution were placed in 7 × 70 mm test tubes and mixed. Optical density was read against a reagent blank at 565 mμ in a Beckman DU spectrophotometer adapted for ultramicro measurements as described by Lowry and Bessey.[9] Fused silica micro cells with 10-mm light path were used. Additional readings were made at 66 mμ and 420 mμ in order to compensate for hemolysis and lactescence when present.

Cephalin flocculation and icteric index were determined by the ultramicro technique of Caraway and Fanger,[2] prothrombin time according to Kato,[8] coagulation time by the capil-

lary tube method, and hemoglobin with a Hellige hemometer. Ammonia tolerance tests[18] were done by administering with a stomach tube, 0.3 ml of ammonium chloride solution containing 867 μg of NH_3-N, followed in 15, 30 and 60 minutes by the withdrawal of heart blood for determination of blood ammonia levels by the method of Nathan and Rodkey.[10]

Electrophoretic studies were carried out on a Spinco model R paper electrophoresis apparatus according to standard techniques. Whatman 3MM filter paper strips and a barbiturate buffer solution (0.1N, pH 8.6) were used. Migration of the protein components was induced by a constant current of 4 ma for 22 hours. Staining was with bromphenol blue and evaluation was made photometrically with the Analytrol, a direct scanning device. The serum samples were obtained from blood collected in capillary tubes from the incised ventral tail vein. Individual samples were used throughout. Total serum protein concentrations were estimated spectrophotometrically.[15]

The perfusion method of Yolles *et al.*,[19] was used for recovery of adult worms from the portal and mesenteric veins. Liver egg counts were made by a digestion technique. The weighed liver was blended in 35 ml of physiologic saline for 15 seconds in a Waring Blendor and quantitatively transferred to a 400-ml flask previously calibrated at 250 ml. Pepsin digest solution* was added to bring the volume to 250 ml. The stoppered flasks were held at 37°C for 48 hours to allow for digestion. When the tissues were digested the eggs were suspended in the digest medium by brisk agitation and 1 ml aliquots were transferred to Sedgwick-Rafter cells and the eggs counted under the microscope. Counts were made on 2 aliquots for each digest preparation.

Histological evaluation was made on tissue sections stained with hematoxylin and eosin and with Masson's trichrome stain.

RESULTS

Four hundred and fifty mice in 3 groups were exposed to *S. mansoni* and numbers of infected mice along with suitable controls were necropsied at 2-week intervals from the sixth to the 16th week thereafter for the purpose of following the pathological changes, growth of the worms and accumulation of parasite eggs in the liver. Determination of portal pressure and study of blood and liver function were made at these times also.

* 3 gm pepsin (1:10,000), 5 gm NaCl, 15 ml HCl, H_2O to 1,000 ml.

ANATOMICAL OBSERVATIONS

1. Gross anatomy. Data obtained on anatomic changes associated with the schistosome infection are summarized in Table 1. The infected mice harbored 24 worms (average) per animal and there was no preponderance of either sex. At 6 weeks no gross consequences of the infection were present even though an appreciable number of eggs were already in the livers (average of 895 per gm). By the eighth week the livers showed considerable enlargement and the average egg density had increased to 1,765 per gm. From the eighth week on, there was no measurable increase in egg density. At 10 weeks, in

FIG. 1. Mouse infected 14 weeks; yellow latex injection of portal system. Note: hepatomegaly, splenomegaly, and tortuous periesophageal varix. Latex medium reached the systemic circulation through the collateral pathway, passed through the right side of the heart, and filled the pulmonary artery.

TABLE 1

Necropsy findings on mice infected with Schistosoma mansoni

Weeks after infection	Average weight of mice (gm) ± S.E.*	Average number of worms per mouse ± S.E.†	Average total liver egg count ± S.E.†	Average number of eggs per gm liver	Average liver weight (gm) ± S.E.*	Liver weight as % of body weight	Average spleen index ± S.E. *‡	Proportion of mice with ascites	Proportion of mice showing esophageal varices
0	22.9 ± .7				1.4 ± <.1	6.1	186 ± 8	0/21	0/21
6	21.5 ± .5	18.6 ± 2.7	1340 ± 156	895	1.5 ± <.1	6.9	201 ± 6	0/28	0/28
8	19.4 ± .3	32.3 ± 2.6	3350 ± 709	1765	1.9 ± <.1	9.8	202 ± 7	0/28	0/28
10	23.0 ± .3	30.5 ± 2.0	4250 ± 950	1700	2.5 ± <.1	10.9	245 ± 7	3/28	12/28
12	22.8 ± .3	16.3 ± 2.5	3705 ± 655	1600	2.3 ± <.1	10.1	242 ± 10	4/28	8/28
14	24.3 ± .4	21.7 ± 3.3	3440 ± 420	1430	2.4 ± <.1	9.9	305 ± 16	5/17	7/17
15	24.4 ± .3	24.3 ± 1.5	3795 ± 906	1650	2.3 ± <.1	9.4	254 ± 11	1/28	10/28

* Based on 17 to 28 animals in each group.

† Based on 8 to 10 animals in each group.

‡ Spleen index was calculated by multiplying the length (mm) by width (mm).

FIG. 2. Control mouse. Yellow latex injection of portal system. The latex medium did not reach the systemic circulation.

addition to liver enlargement, splenomegaly, esophageal varices, and ascites were present, and from that time on, all of the above mentioned pathologic changes were observed consistently.

The esophageal varices arose from the gastric or splenic vessels and terminated either in the vena cava or in the pulmonary vessels (see Figs. 1 and 2). In most cases there was a single tortuous vessel passing along the serosal surface of the esophagus. The vessels were periesophageal in every case and were never seen within the wall or on the mucosal surface. No evidence of hemorrhage from the esophageal varices was observed.

2. Microscopic anatomy. After 6 weeks of infection the livers showed characteristic granulomatous lesions about the schistosome eggs. Most of the areas of reaction were in the early stage of development and showed endothelial proliferation and considerable leucocytic infiltration. In older infections, the lesions had progressed to the scar stage in which the cellular elements had either disappeared or were reduced in number. The connective tissue formation around the schistosome eggs remained localized and showed no tendency to invade the surrounding parenchyma. In addition to the granulomatous lesions produced by the eggs, there was some accumulation of mononuclear and polymorphonuclear cells in the periportal spaces; however, there was no indication that the reaction in these areas ever led to connective tissue proliferation. No dead worms were ever observed in the portal vessels of the infected livers. There seemed to be no question but that the most significant histopathologic changes in the liver were associated with the invasion of the portal canals by the schistosome eggs.

TABLE 2

Observations on physiologic changes due to Schistosoma mansoni *infection*

Weeks after infection	Portal pressure (cm H_2O) ± S. E.*	BSP† retention (%) ± S.E.*	Cephalin flocculation test*	Icteric index ± S.E.*	Prothrombin time (sec.) ± S.E.*	Hemoglobin (gm%) ± S.E.*
0	5.0 ± .7	5.8 ± .9	negative	5.4 ± .5	15 ± 1	15.4 ± .1
6	6.3 ± .4	9.0 ± 1.0	negative	6.1 ± .5	18 ± 1	15.1 ± .1
8	9.4 ± .4	18.7 ± 2.9	negative	3.4 ± .3	17 ± 1	12.2 ± .4
10	10.3 ± 1.1	16.4 ± 2.7	negative	4.2 ± .5	21 ± 1	8.9 ± .8
12	11.9 ± 1.2	18.0 ± 1.0	negative	3.4 ± .4	22 ± 1	11.7 ± 1.0
14	10.4 ± 1.0	18.5 ± 4.7	negative	5.8 ± .4	23 ± 1	8.9 ± .9
16	9.4 ± .8	12.4 ± 1.8	negative	4.7 ± .3	22 ± 1	9.5 ± 1.2

* Based on 8 to 10 animals in each group.

† Bromsulfalein.

TABLE 3

Ammonia tolerance tests run 13 weeks after exposure to S. mansoni. *Each value was obtained from a separate mouse*

Mice	Blood ammonia levels (μg %)			
	0 min.	15 min.	30 min.	60 min.
Uninfected mice	62	404	128	136
	30	166	250	410
	36	444	142	410
Average	43	338	173	318
Infected mice	127	1142	255	576
	157	320	636	389
	428	4480	832	316
Average	237	1981	575	427

The spleens from the infected mice only rarely showed schistosome eggs; the histological picture was that of congestion, showing dilatation of the sinusoids but no evidence of fibrosis.

PHYSIOLOGIC OBSERVATIONS

Data obtained on certain physiologic changes associated with the schistosome infection are summarized in Tables 2, 3 and 4.

Portal hypertension (*Table 2*). The average portal blood pressure in uninfected animals was 5.0 ± 0.7 cm of water. In infected mice the portal pressure rose to an average of 11.9 ± 1.2 cm of water at 12 weeks and remained at a high level through the 16th week. Portal pressures as high as 18.5 cm of water were recorded in some of the infected mice.

Bromsulfalein retention test (*Table 2*). Fifteen minutes after the injection of 1.2 mg of bromsulfalein dye in 0.3 ml of saline, uninfected mice retained an average of 5.8 ± 0.9 per cent of the dye. In infected mice, the dye retention reached 18.7 ± 2.9 per cent by the eighth week and remained elevated through the 16th week of infection.

Ammonia tolerance test (*Table 3*). The baseline blood ammonia level in mice infected for 13 weeks averaged 237 μg per cent as compared with 43 μg per cent in uninfected control mice. Fifteen minutes after giving the test dose of ammonium chloride by stomach tube, the blood ammonia level averaged 1981 μg per cent in infected and 338 μg per cent in control mice. After 30 minutes the levels averaged 575 and 173, and after 60 minutes 427 and 318 μg per cent, respectively.

Cephalin flocculation test (*Table 2*). The cephalin flocculation tests performed at 2-week intervals from the sixth to the 16th week were consistently negative.

Icteric index (*Table 2*). The average icteric index obtained on a group of 10 uninfected control mice was 5.4 ± .5. The findings on the infected mice were not significantly different from those on the controls.

Electrophoretic analysis of serum (*Table 4*). The fractionation of the serum proteins by zone electrophoresis showed that in infected mice the relative distribution of the various components was considerably altered. The changes in the electrophoretic pattern were due mainly to a marked increase in the slow moving globulin components and a relative decrease in albumin. Based on the absolute concentration of the serum components, however, it is seen that

TABLE 4

Zone electrophoresis of serum proteins. Standard errors of the means are shown in parenthesis

Experimental group	No. of mice	Total protein (gm%)	Albumin (%)	Globulins (%)				Albumin (gm%)	Globulins (gm %)				Albumin globulin ratio
				Alpha$_1$	Alpha$_2$	Beta	Gamma		Alpha$_1$	Alpha$_2$	Beta	Gamma	
Control:													
6 weeks	11	5.5	55	7	7	19	12	3.0	.4	.4	1.0	.7	1.2
		(.1)	(1.0)	(.4)	(.7)	(.9)	(1.0)	(.07)	(.06)	(.06)	(.07)	(.06)	(.05)
16 weeks	8	6.1	53	8	7	21	11	3.2	.5	.4	1.3	.7	1.2
		(.1)	(1.3)	(.6)	(.6)	(1.2)	(.8)	(.16)	(.03)	(.05)	(.04)	(.08)	(.08)
Infected:													
6 weeks	9	6.3	53	8	7	18	14	3.3	.5	.4	1.1	1.0	1.1
		(.3)	(1.6)	(.6)	(.3)	(.7)	(1.1)	(.22)	(.05)	(.02)	(.04)	(.10)	(.08)
8 weeks	8	7.7	45	8	7	21	19	3.5	.6	.5	1.6	1.5	.9
		(.2)	(.2)	(.5)	(.1)	(.5)	(1.1)	(.09)	(.04)	(.03)	(.11)	(.15)	(.07)
10 weeks	10	8.9	42	6	7	23	22	3.7	.5	.6	2.1	2.0	.8
		(.3)	(1.2)	(.3)	(.3)	(1.1)	(2.2)	(.15)	(.02)	(.03)	(.06)	(.15)	(.04)
12 weeks	10	9.0	42	7	6	19	26	3.8	.6	.5	1.7	2.4	.8
		(.4)	(2.1)	(.6)	(.4)	(2.1)	(2.8)	(.23)	(.04)	(.04)	(.21)	(.09)	(.08)
14 weeks	8	9.5	39	6	7	23	25	3.7	.6	.6	2.2	2.4	.7
		(.4)	(1.8)	(.5)	(.4)	(1.6)	(1.4)	(.19)	(.05)	(.04)	(.20)	(.25)	(.05)
16 weeks	8	10.0	38	4	6	27	25	3.8	.4	.6	2.7	2.5	.6
		(.4)	(2.0)	(.5)	(.5)	(2.1)	(2.2)	(.23)	(.05)	(.05)	(.19)	(.18)	(.05)

there was a progressive increase in the beta and gamma globulins and little or no change in the albumin and alpha globulin concentrations. The uncompensated rise in the globulin fractions resulted in a sharp increase in the total protein concentration and a reversal in the albumin-globulin ratio.

Prothrombin time (*Table 2*). The average prothrombin time obtained on a group of uninfected control mice was 15.1 ± 1 sec, and on infected animals there was a small but statistically significant increase in prothrombin time at the various time intervals studied between the sixth and 16th week of infection.

Hemoglobin (*Table 2*). Repeated hemoglobin determinations on infected mice showed that an anemia developed by the 10th week of infection. In some of the animals, the hemoglobin levels fell as low as 4 to 6 per cent.

DISCUSSION

Despite Symmers'[14] observation (in 1903) of *S. mansoni* in the liver of a patient who died of severe liver disease, it was many years before the syndrome of hepato-splenic schistosomiasis was recognized. It is now generally conceded that the prominent sign of an enlarged spleen in hepato-splenic schistosomiasis, that was so confusing in the past, is due mainly to congestive enlargement (Banti's syndrome) resulting from blockage of the portal blood flow in the liver.

The human patient with the hepato-splenic form of schistosomiasis exhibits 5 primary signs; hepatomegaly, splenomegaly, esophageal varices, ascites and anemia. The syndrome that developed in our mice infected with *S. mansoni* bore a striking resemblance to this human syndrome and was characterized by the same 5 signs. Hepatomegaly developed by the eighth week and was followed with the appearance of splenomegaly, esophageal varices, ascites, and anemia by the 10th week. In spite of all of the above mentioned consequences of the infection, there is little evidence of altered liver function. The results of the liver function tests suggest that the liver parenchyma suffered but little damage due to the schistosome infection and that the elevated bromsulfalein retention and the higher blood ammonia levels observed in the infected mice were most likely due to diversion of a significant portion of the normal portal blood flow into the shunt(s) leading to systemic circulation, thereby bypassing the liver. The total work done by the liver cell, as it is readily seen, is dependent not only on its

functional capacity, but also on the load with which it is presented. Thus, the rate of removal of ammonia and bromsulfalein dye from the blood stream of the infected mice was limited probably more by changed portal circulation than by any cellular disease. In this connection, the results of the serum electrophoretic analyses are of interest. In the infected mice it was found that normal albumin levels were maintained, even though there was considerable alteration in the concentrations of the slower-moving globulin components. Since the site of origin of albumin is considered to be limited to the the liver parenchyma, it would appear that the synthesizing ability of this structure was not seriously damaged. The globulin components, on the other hand, are thought to originate mostly in the reticuloendothelial system. Thus it seems to be clearly indicated that portal hypertension and its consequences are the main attributes of the disease.

The results of the present study tend to demonstrate that the pathogenesis of hepato-splenic schistosomiasis in the mice was due primarily to the penetration of the portal canals of the liver by large numbers of schistosome eggs with subsequent formation of granulomata, and is probably not due to other factors, such as diet, toxins produced by the worms or the presence of dead worms in the liver. Malnutrition, which has been thought by some to be the main etiologic agent, played no significant role in the present study as the animals were provided with a complete, balanced diet.[3] Also, as was shown in earlier work[4] mice infected with *S. mansoni* can digest and absorb dietary fats and proteins almost as well as uninfected control mice. The role of toxins in producing the syndrome can be discounted because they would be diffusely attacking the parenchymal cells and would almost undoubtedly cause severe liver function test derangement. The only hepatic lesion that conceivably could have been sufficient to produce the syndrome observed was the granulomatous reaction about the large number of schistosome eggs trapped in the portal spaces.

Now that the complete syndrome of hepato-splenic schistosomiasis has been experimentally produced in a laboratory animal and has been found to resemble very closely the syndrome that occurs in humans, it will be possible to study more fully the consequences of this parasitic infection under controlled laboratory conditions. Such studies should yield not only specific information concerning schistosomiasis but also much valuable information concerning the role of intrahepatic blocking agents in the production of liver disease.

SUMMARY

A syndrome that closely resembles hepato-splenic schistosomiasis in humans developed in mice following infection with *Schistosoma mansoni*. Data obtained from the infected mice suggest that the pathogenesis of the syndrome was related to the penetration of the portal canals of the liver by large numbers of schistosome eggs which incite granulomatous reactions and obstruction of portal blood flow. This in turn produces portal hypertension and the development of congestive hepato-splenomegaly, esophageal varices and ascites. Histologically there was little damage to the liver parenchyma, which is further borne out by very little liver function test derangement. A severe anemia developed in some of the infected mice (4 to 6 gm per cent hemoglobin).

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MALARIA SURVEILLANCE IN THE UNITED STATES, 1956–1957

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Surveillance of malaria during the years 1956–1957 has revealed the extent to which the goal of eradication has been achieved in the United States.[1-4, 8, 11, 13] While occasional fully confirmed cases of primary indigenous malaria still appear in this country, the magnitude of the decline in malaria morbidity over the past 22 years is apparent upon inspection of annual totals of reported cases of all types. In 1935 a total of 150,000 cases were reported while in 1945 the figure was 60,000. By 1955 reported cases had fallen to 522, and in 1957 an historic low of 132 cases was reached.[10] In the present report the malaria surveillance findings for 1956 and 1957 in the United States are summarized. Three small foci of primary indigenous malaria are described in detail. One of these, in eastern Oklahoma, investigated by the authors, appears to have been a surviving endemic focus of malaria. Two other clusters of cases reported from California demonstrate that reintroduction of the parasite into mosquito populations can still occur.

As malaria declines toward the vanishing point in the United States the need for a continuing national surveillance persists. The vector continues to be present in abundance in many areas, and the risk of parasite introduction from abroad is constantly present. While the possibility of establishment of endemic foci exists, the means are at hand to eliminate them as they occur. Through the Malaria Surveillance Program the Communicable Disease Center (CDC) endeavors to recognize potentially dangerous situations promptly and to help initiate appropriate control measures.

The Surveillance Section of CDC depends for its information on three basic sources: the state health departments, the Morbidity and Mortality Weekly Reports prepared by Dr. Carl Dauer of the National Office of Vital Statistics, and the National Repository for Malaria Slides, directed by Dr. Marion Brooke, in the Laboratory Branch of CDC at Chamblee, Georgia. Information about malaria in military personnel is received directly from state epidemiologists who receive reports of military cases occurring within their jurisdictions. An active correspondence is maintained with state epidemiologists concerning reports of malaria cases, and case evaluations are generously supplied by the states when they become available. States submit malaria slides to the National Repository for confirmation of diagnosis, and reports of such confirmations come to the Surveillance Section. The data collected by these methods may, at times, be supplemented by field investigations in cooperation with state health authorities when such assistance is invited. All data are summarized in a Surveillance Report which is published and distributed periodically to the states and to others concerned with malaria control activities.[7]

Reported cases are classified into the following categories to the extent that available data permit.

Diagnosis:

Confirmed—adequate clinical, laboratory and epidemiologic evidence.
Presumptive—clinical and epidemiologic data consistent but laboratory evidence in doubt.
Not confirmed—insufficient data available.
Not malaria—sufficient evidence of other diagnosis.

Origin:

Indigenous—epidemiologic evidence indicates origin within the United States.
Foreign—evidence that infection was acquired abroad.

A substantial number of cases are reported from military sources, most among returnees from Korea, but a few among those returning from other malarious areas. Reports from the armed services are accepted as confirmed without submission of explicit data because of the continuing thoroughness of the study that these cases receive. In the civilian cases every effort is made to develop a complete record including laboratory confirmation. In the final analysis

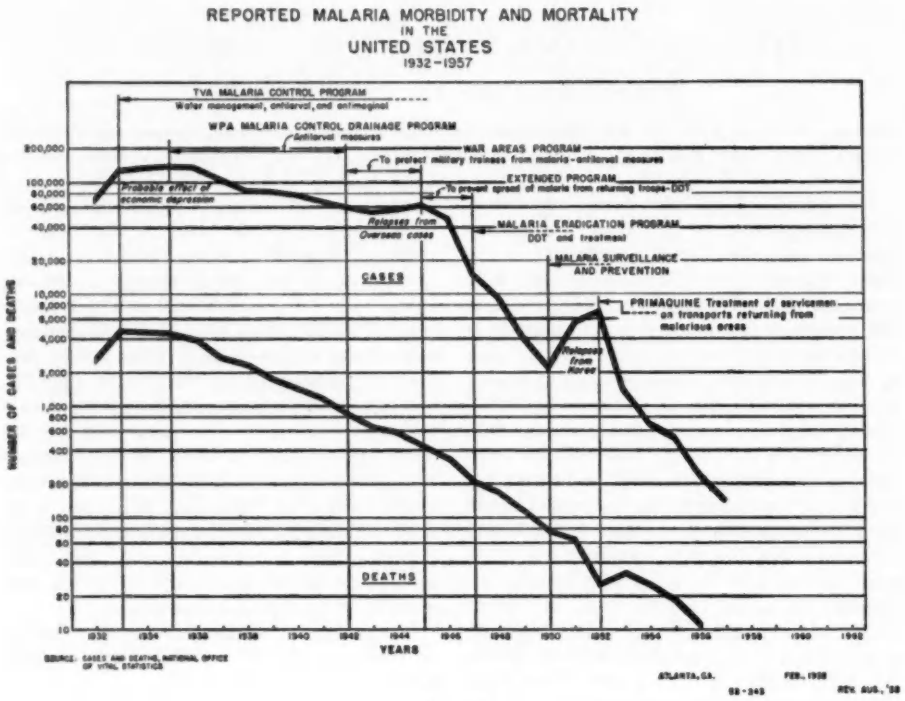

FIG. 1

TABLE 1

Malaria surveillance in the United States, 1956–1957

	1956	1957
Total reports to NOVS*	234	132
Total reports to CDC†	238	157
Appraised by CDC	198	138
Confirmed	97	102
Presumptive	32	6
Not confirmed	67	25
Not malaria	2	5
Total confirmed and presumptive	129	108
Indigenous civilian	14	11
Imported military	46	56
Imported civilian	52+	41

* National Office of Vital Statistics.

† Several states do not regularly report military cases, and cases clearly of foreign origin, to NOVS. These are, however, reported to CDC.

the diagnosis and classification of the case remains the decision of the state epidemiologist.

The reported incidence of malaria cases and deaths from 1932 to 1957 are shown in Figure 1. This is an extension of the figure first presented by J. M. Andrews.[2] The numbers of reported cases continued to fall during 1956 and 1957. The malaria deaths, a notoriously poor index of malaria in this country[4, 5, 9] declined to essentially the vanishing point by 1956.

The results of the 1956–1957 malaria surveillance program are summarized in Table 1.

DISTRIBUTION OF REPORTS WITHIN THE UNITED STATES

During 1956 CDC received reports of 238 cases of malaria from 34 states and the District of Columbia. Approximately half of the listed cases were reported from Texas and California. The only other states to report more than six cases were Oklahoma and Florida. The 129 confirmed or presumptive cases came from 23 states and the District of Columbia. These states are indicated in Figure 2.

In 1957 the 157 case reports received by CDC came from 30 states. Malaria was reported from all over the United States rather than primarily from the once traditionally malarious states.

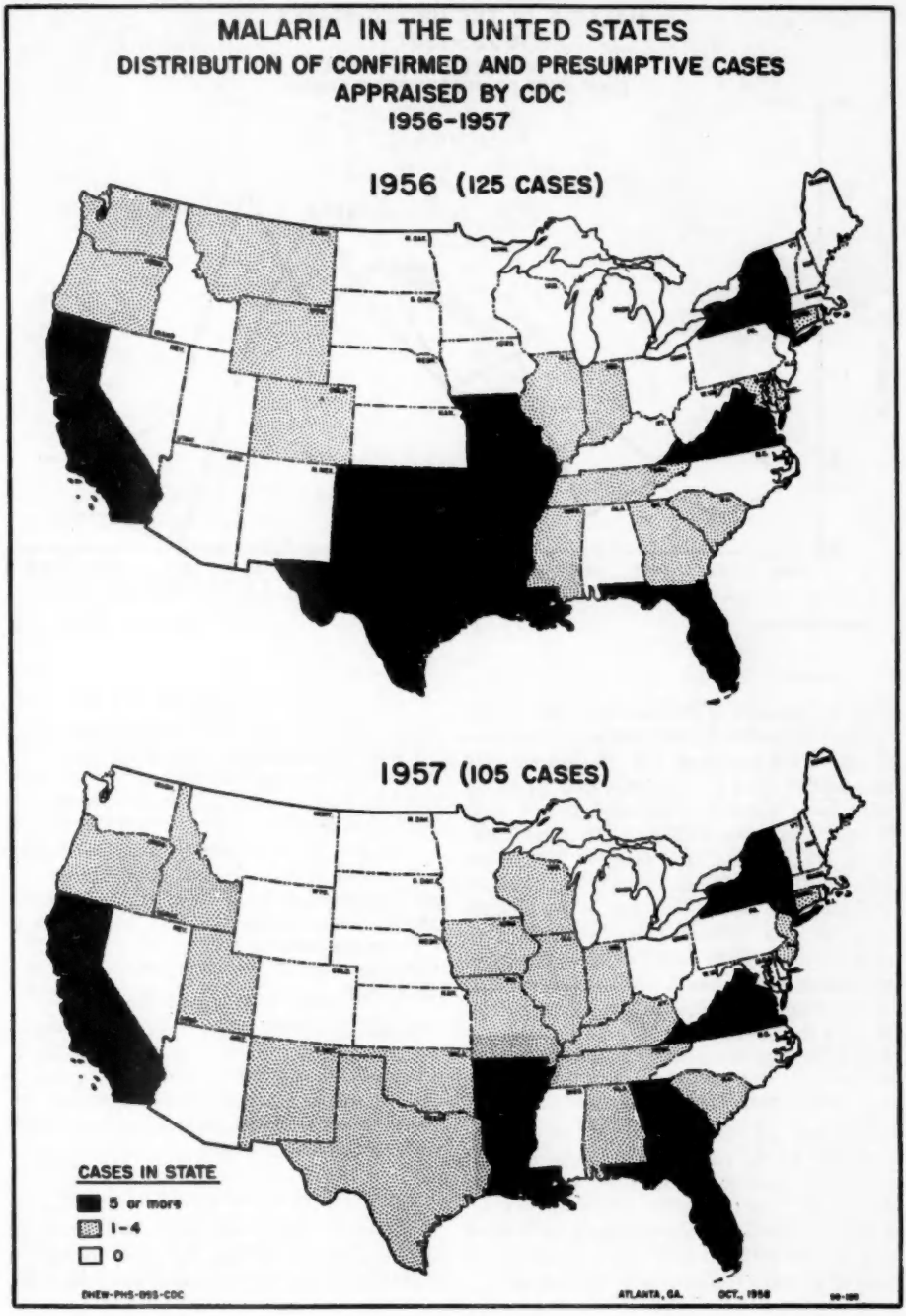
MALARIA IN THE UNITED STATES
DISTRIBUTION OF CONFIRMED AND PRESUMPTIVE CASES
APPRAISED BY CDC
1956-1957
1956 (125 CASES)
1957 (105 CASES)
CASES IN STATE
5 or more
1-4
0
DHEW-PHS-BSS-CDC
ATLANTA, GA. OCT., 1958

FIG. 2

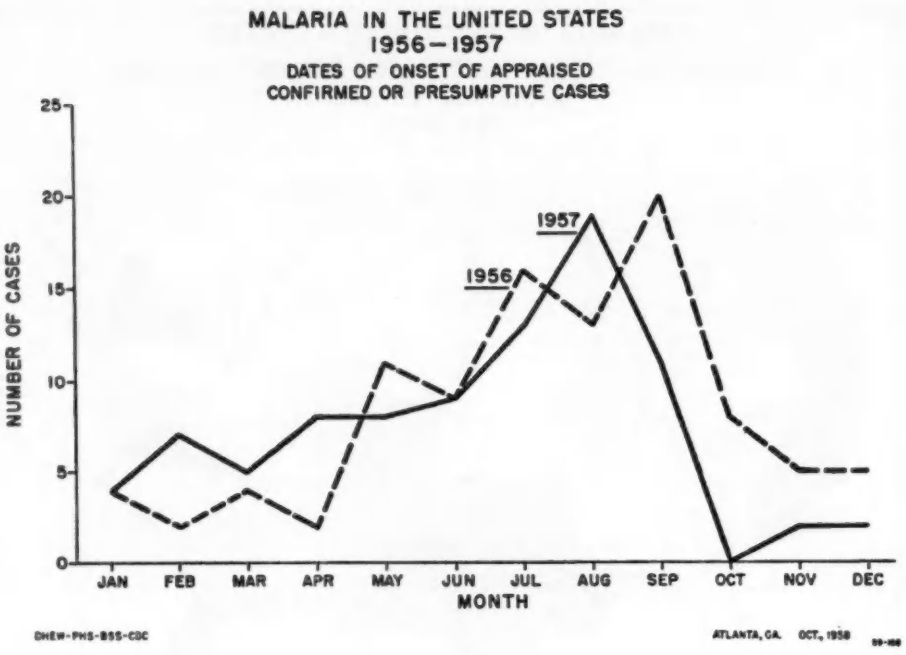

FIG. 3

Since the majority of the cases have their origins overseas this rather broadly scattered occurrence of cases is not surprising. The 108 confirmed and presumptive cases were reported from 24 states. Only seven states reported more than 5 cases in 1957. These were California (36 cases), Oklahoma (15), Florida (15), Georgia (13), Texas (10), New York (9) and Virginia (8). All of the Virginia cases and all but two of the Georgia cases were in military personnel. New York reported two military cases and seven in persons who had travelled abroad. The most conspicuous change from 1956 was in the report from Texas where a drop from 62 to 10 cases probably reflected a change in reporting procedures.

TABLE 2

Malaria in the United States, 1956–1957. Origin of confirmed and presumptive cases

Origin	1956		1957	
	Number	Per cent	Number	per cent
Military overseas	46	41	56	52
Mexico (migrant laborers and others)	32	29	16	15
Other foreign	20	18	25	23
Indigenous	14	12	11	10
Totals	112	100	108	100

SEASONAL DISTRIBUTIONS

The date of onset of clinical malaria was available for 99 cases in 1956. The seasonal morbidity peak for these cases was reached during the July-September period (Figure 3). A similar pattern with a peak during the summer months was noted in 1957. Since about 90 per cent of all cases reported in both years were of foreign origin this seasonal concentration appears to be unrelated to any ecologic factors within the United States.

LABORATORY DATA

Plasmodium vivax was responsible for 67 of the 78 infections in 1956 for which we have a definite diagnosis. Seven *Plasmodium falciparum*,

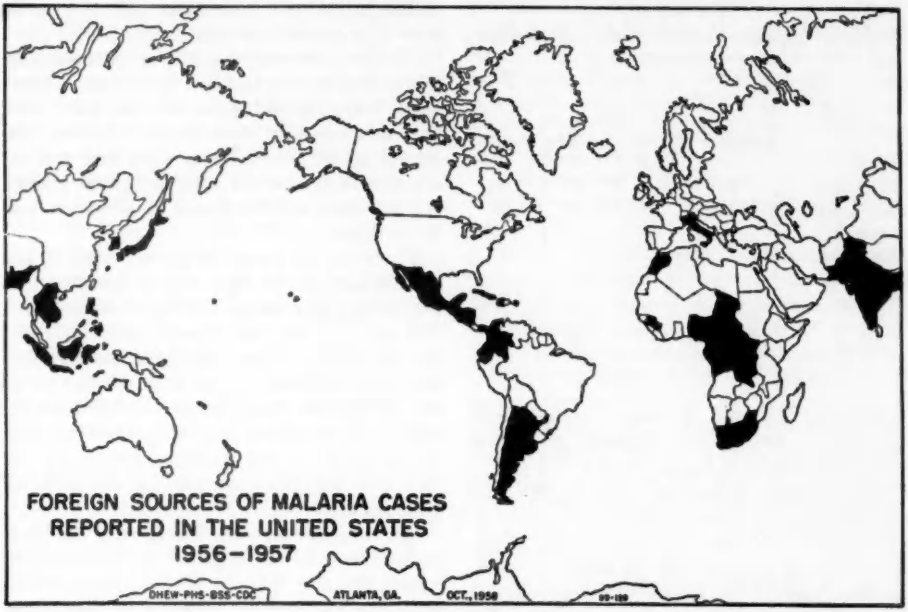

FIG. 4

3 *Plasmodium malariae*, and 1 mixed *P. vivax-P. falciparum* infection were reported.

In 1957 *P. vivax* produced 67 of the 74 infections for which we have laboratory information. Four additional infections were due to *P. falciparum*, one to *Plasmodium ovale* and two to a "rare ring type, not identified." In both years transfusion-induced malaria was a very rare event. Several cases may have been related to transfusions but no fully substantiated instance came to our attention.

COUNTRIES OF ORIGIN

Table 2 summarizes the information obtained for the two years concerning the geographic origins of cases. In both years the majority of the military personnel with malaria had acquired their infections in Korea. A high proportion of the civilian cases of foreign origin originated in Mexico, about half occurring among migrant laborers and the rest in persons who had travelled in the country. In 1956 cases of Mexican origin outnumbered all other civilian cases of foreign origin combined, but in 1957 cases from other foreign sources exceeded those originating in Mexico, perhaps reflecting both improvements in malaria control in Mexico and in border control of migrant laborers entering the United States. The majority of the civilian cases imported from abroad in 1957 were in tourists, missionaries and foreign exchange students. In 1956 approximately 26 foreign countries or areas were considered to have been source areas of infections; in 1957 this number was 23. Figure 4 indicates countries that were identified as probable sources of confirmed and presumptive cases of malaria in 1956–1957.

INDIGENOUS MALARIA IN 1956 AND 1957

The primary indigenous cases for each year are presented in Table 3 under two general headings: those cases for which evidence of indigenous origin is conclusive, and those for which possible indigenous origin cannot be ruled out. The majority of the cases in the latter category are laboratory confirmed but not supported as indigenous by sufficiently sound epidemiologic evidence. A few cases in this group, however, cannot be considered conclusively

TABLE 3

Primary indigenous malaria in the United States, 1956–1957

State	1956		1957	
	Conclusive	Possible	Conclusive	Possible
Oklahoma	4	—	2	2
California	3	3	3	3
Texas	—	2	—	—
Tennessee	—	1	—	—
Missouri	—	1	—	—
Indiana	—	—	—	1
Totals	7	7	5	6

indigenous, in spite of sound epidemiologic associations, because the diagnosis of malaria was not confirmed in the laboratory.

All conclusive indigenous cases in 1956 and 1957 occurred in three small foci or clusters in two states, Oklahoma and California. These three clusters of cases will be discussed below in some detail. These discussions will be followed by a brief consideration of the group of possible indigenous cases.

Sacramento County, California—1956

During the fall of 1956 three confirmed primary indigenous cases occurred in Sacramento County in the Hood-Clarksburg area. Epidemiologic investigations of these cases were conducted by Dr. Rosemary Brunetti of the Sacramento County Health Department. At the time that these cases occurred there were between four and five thousand Mexican agricultural workers in the immediate area. There was a large turnover of these workers, and at least two were reported by local physicians to have had malaria while in the county, although these cases were not confirmed by health authorities. One of these cases was in a worker who arrived in the area August 9 and became ill soon thereafter; the other who came in late August became ill September 1. The investigator concluded that the former of these workers may well have been responsible for the introduction of the parasite into the local anopheline population that resulted in the indigenous cases. Studies revealed that *Anopheles freeborni* were present in moderate numbers in the region in August and September. Residents in Hood complained of the mosquitoes and few homes were equipped with screens.

The first case was in an 18-year-old male high school student who had been a resident of Hood since 1944. He had come at that time from Colorado, and had never been to Mexico. He became ill on September 2, 1956, and was diagnosed September 12 after a typical course. His infection was confirmed as vivax malaria by the state.

The second case was also a Hood resident, the grandfather of the high school student. The boy and the grandfather lived in the same house. This man was 70 years old and unable to work. He had lived in Hood since 1944 and had also come from Colorado at that time. He had never been in Mexico though he was of Mexican ancestry. He developed a typical illness on September 6, which was diagnosed September 12. This case too was confirmed by the state as vivax malaria.

The third Sacramento County case was in a 26-year-old male schoolteacher in Clarksburg, a community only a short distance from Hood. This man had recently returned to Clarksburg from two years residence in New York, but he was a native of Sacramento County.

While working around his school building between September 4 and 7 he was bitten by many mosquitoes and on September 29, he became ill. His illness continued intermittently until November 17, when the diagnosis of vivax malaria was made at a hospital laboratory. Although this case was not confirmed by the state, the investigators considered it to be strongly presumptive and epidemiologically associated with the other two confirmed indigenous cases in nearby Hood.

Sutter County, California—1957

During August 1957 four cases of malaria were reported from Sutter County.[6] Three of these cases were confirmed as vivax malaria by the state laboratory; one, in a 19-year-old male who had arrived from Missouri 3½ weeks before onset of illness, was never satisfactorily confirmed by the state and has been placed in the "possible" column for 1957 in Table 3. The three confirmed cases were in males aged 53, 65 and 16 who were neighbors on a ranch in a region of fruit orchards. None of the group had had military service. Two of them had been in

the county for at least two months. The third man had apparently resided in the county for a longer period of time. He claimed to have had malaria in Texas and Oklahoma 30 to 40 years ago. The dates of onset of illness for the three were August 2, August 2 and August 3. All three cases have been accepted as confirmed primary indigenous malaria by Dr. A. C. Hollister, Jr., Chief of the Bureau of Acute Communicable Diseases of the California State Department of Public Health.

Anopheles freeborni were present at the time in sufficient numbers to transmit the infection. A farm labor camp for Mexican Nationals was located about one-quarter of a mile from the ranch where the cases occurred, but it was not demonstrated that anyone at this camp was the source of the infection.

Cherokee County, Oklahoma—1956–1957

In 1956 four cases of vivax malaria were reported to the Oklahoma State Health Department from Cherokee County and confirmed by the state laboratory. In 1957 two more cases from this one county were reported to and confirmed by the state. These reports resulted in a cooperative field investigation in April 1958 by the Oklahoma State Health Department and the Epidemic Intelligence Service of CDC. As a result of these studies the six confirmed cases were found to be primary indigenous malaria. Two additional strongly presumptive indigenous cases were detected in the county during the investigations.

Cherokee County, in the northeastern part of the state, is the seat of the Cherokee Indian Nation. A large proportion of the population is Indian. The countryside is hilly and cut by many streams and side valleys. Several major rivers traverse the county. Many of the Indians live in small cabins hidden away in the side valleys or "hollows;" the general economic position of the rural population is not strong. The major town, Tahlequah, with a population of 6,000 is located near the geographic center of the county. It was at the Public Health Service Indian Hospital located here that the six cases, all of which were in Indians, were hospitalized and diagnosed initially. Each of the cases presented with a characteristic picture of vivax malaria.

The malaria cases occurred in two foci near Tahlequah. One focus was on the banks of the Illinois River; the other along a stream in a small hollow not far from the main river. There had been moderately heavy anopheline breeding every year in many parts of the county, including the areas along the river. There had been no organized mosquito control activities in the area for some years, and few of the homes in this area were well screened. Before World War II, malaria had been conspicuous in the region.

Three of the 1956 cases, children aged 8, 12 and 13 of the same family, were probably infected at their home just above the sloughs of the Illinois River in the summer or fall of 1955. They attended a vocational school several miles south of Tahlequah during the winter months and did not leave the school, except for a brief December vacation at home. They became ill in late April–early May of 1956, with onsets of symptoms within a few days of each other. These three siblings were the only children at the school who developed malaria (other cases were actively sought for).

Two of the other three cases were in adult females, aged 21 and 33, who were probably also infected by mosquitoes beside the Illinois River sloughs. One became ill May 1, 1957; the other April 22, 1957. While it is likely that latency is the explanation for the dates of clinical onset of illness in the three children, this is less certain for the two women.

The final laboratory confirmed case occurred in a 7-year-old boy who lived in a poor shack in a small hollow east of the Illinois River. He became ill August 26, 1956. During investigations in this hollow two other inhabitants, a father and son, who were next door neighbors of the boy with the confirmed infection gave convincing histories of similar illness about the time when the boy became ill. Neither of these individuals with presumptive cases sought medical attention at time of illness nor was either case laboratory confirmed. Both responded to a self-administered proprietary antimalarial preparation.

Investigation failed to reveal any source responsible for recent introduction of the parasite into the mosquito population. Foreign travellers and returned veterans were considered as possible sources and ruled out fairly conclusively. None of those who had been ill had ever travelled far from their homes. Migrant laborers do not come into this area. Although

it could not be established as a certainty it appeared possible that the cluster of cases in this county represented malaria that had perpetuated itself for at least several years. Since the investigations there has been an active mosquito control program in the county and additional cases have not been reported.

The Possible Indigenous Cases

1956

California—Three isolated cases in civilians. One of these cases which was diagnosed in Sutter County was appraised by the state as primary indigenous. The other two cases occurred in persons who had recently arrived in California from Alabama and Arizona. Possibly of primary indigenous origin in these states.

Texas—Two single cases of confirmed vivax malaria in civilians. These cases were not in epidemiologic association with each other. For lack of evidence to the contrary they were appraised as primary indigenous malaria.

Tennessee—A confirmed vivax case in an 8-year-old girl from Lake County appraised as possible primary indigenous malaria. No other cases were reported from the area.

Missouri—A case in a 7-year-old girl which was not laboratory confirmed. Considered presumptive primary indigenous malaria. No other cases were reported from the area.

1957

California—1. A case in a 56-year-old Colusa County male which was not laboratory confirmed by the state. Considered presumptive and possibly primary indigenous, for lack of evidence to the contrary.

2. A 78-year-old male who developed confirmed vivax malaria in March 1957 in San Diego County. He had received several transfusions in preceding months. Transfusion considered probable source; otherwise must be considered indigenous.

3. A Sutter County case in a 19-year-old male which was not laboratory confirmed, but presumptive and in epidemiological association with a confirmed cluster of cases occurring in August 1957.

Indiana—A case in a 13-year-old male without laboratory confirmation. He visited in Arkansas for a week in August 1956. Onset of illness in April 1957. Considered presumptive and possibly primary indigenous to Arkansas.

Oklahoma—A husband and wife, aged 50 and 39, both of whom developed confirmed vivax malaria in the spring of 1957, onsets May 28 and April 20. Longtime residents of Guthrie, Oklahoma who had travelled out of the area only to Hot Springs, Arkansas in January 1957. Considered highly probable primary indigenous malaria.

DISCUSSION

In 1957, as cases of malaria approached the vanishing point, the evidence indicated that the goal of eradication of malaria in the United States had not yet been achieved although it was near at hand. While indigenous cases continued to occur, it was probable that most, though perhaps not all, resulted from recent reintroductions of the plasmodium into the mosquito population from foreign sources.

The recent decline in malaria morbidity in this country undoubtedly reflects in part the results of intensive worldwide antimalarial campaigns.[12] Widespread use of curative antimalarial drugs among returning overseas military personnel has helped to alter the situation that prevailed in the first year of the Korean War when hundreds of infected troops returned to the United States. Another factor contributing to the recent decline is a change in attitude in several states in regard to case reporting. Most states now accept for purposes of national reporting only those cases for which reliable epidemiologic and laboratory data are available. Case reporting by physicians has varied through the years. Malaria has been both grossly under-reported and over-reported at various times in the past.[4] The fact that, of 157 cases in 1957, 108 were confirmed or at least considered presumptive demonstrates that greater conservatism and accuracy has now become the rule.

The definite seasonal peak for reported malaria during the July–September period each year appears not to be a reflection of anopheline and plasmodium activity in this country since over 90 per cent of our cases are of foreign origin. The seasonal peak is probably the resultant of such factors as the summer seasonal increase in U.S. tourist travel abroad, the late summer arrival in this country of foreign exchange students, and summer influxes of migrant laborers from Mexico.

The largest single group of malaria cases continues to be military personnel. In 1956, 41 per cent of all confirmed and presumptive cases occurred in members of the Armed Forces, whereas in 1957 the figure rose to 52 per cent.

Cases originating in Mexico are gradually becoming fewer in number but still exceed those from any other nation except Korea. In 1956, 29 per cent of all confirmed and presumptive cases originated in Mexico; in 1957 the figure had dropped to 15 per cent. All other foreign countries accounted for 18 per cent of the cases in 1956 and 23 per cent in 1957.

Twenty-five cases of probable or possible primary indigenous malaria were identified during the two-year period. In 1956 they represented 12 per cent, and in 1957, 10 per cent, of the confirmed and presumptive cases. Most of the indigenous cases appear to represent reintroductions of the parasite from foreign sources. Only one indigenous situation, that in Cherokee County, Oklahoma, seems possibly to have been an endemic survival rather than a recent reintroduction.

The importance of rapid case finding and immediate field investigations for the continuing control of malaria in this country must be re-emphasized. Until worldwide eradication campaigns advance considerably farther we cannot expect eradication of malaria from this country. The anopheline population in the United States is abundant and our population is totally susceptible to malaria. The use of curative antimalarials must be strongly recommended for all members of the Armed Forces and other travellers returning from malarious areas overseas. Individual case surveillance techniques similar to those used for smallpox and plague, however, offer the best means to prevent serious malaria situations from arising in this country.

SUMMARY

Malaria surveillance data for 1956 and 1957 have been presented and discussed. Points worthy of particular note include the steady decline in morbidity to a very low yearly figure, the appearance of a few primary indigenous cases every year, the importance of the military population in the present pattern of introductions from foreign sources and the declining importance of Mexico as a source of malaria. Eradication, although near at hand, has not been achieved in this country. Eradication here appears to be dependent on at least much more effective control, if not eradication, of malaria elsewhere in the world. For the present, continuing individual case surveillance offers the best means of preventing flare-ups of malaria in the United States.

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THE RENAL RIDGE AS A RELIABLE CHARACTER FOR SEPARATING *TAPHIUS GLABRATUS* FROM *TAPHIUS TENAGOPHILUS**†

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The presence of a renal ridge in *Taphius glabratus* was recorded for the first time by Baker,[1] who considered it a characteristic feature not only of that species, but also of the genus *Australorbis*. That character was recognized by others[14, 16] in *T. glabratus* from Brazil. The last authors, however, observed it to be missing in the closely related species, *Taphius tenagophilus*, listed by Baker[1] under *Australorbis*. Paraense and Deslandes[16] studied the development and histological structure of the renal ridge in *T. glabratus*. Subsequently[11] they confirmed the observations of Pinto and Deslandes[16] on the absence of the ridge in *T. tenagophilus*, and stated (p. 129): "... the ridge and melanic pigmentation along the ventral surface of the renal tube, that we have consistently observed to occur in *glabratus* and to be lacking in *nigricans* ... give support to considering the latter a good species from a taxonomic standpoint (morphospecies)." Crossbreeding experiments showed that the forms with and those without renal ridge correspond to true biological species, separated by absolute reproductive isolation.[8, 12] Moreover, these same authors used the renal ridge as a key character to separate *T. glabratus* from all the other Brazilian species of its genus.[13]

However, some observations, recently published[2, 3, 5, 17] led us to reinvestigate the question of the reliability of the renal ridge as a taxonomic character of *T. glabratus*.

Hubendick,[5] referring to Venezuelan specimens, says (p. 457): "In the species generally called "*Australorbis glabratus* (Say)" all stages from a well developed renal ridge to none at all can be observed."

Rey,[17] after having said that in *T. glabratus* the kidney shows a prominent and pigmented longitudinal ridge, and that in *T. tenagophilus* the kidney is smooth, adds (p. 99): "In some specimens, however, a slight ridge, either unpigmented or poorly pigmented, may be seen in the same situation" (as in *T. glabratus*). No details or illustrations of such a "slight ridge" in *T. tenagophilus* are presented by Rey.

Barbosa and Coelho,[3] dissecting 50 snails from Niteroi, Brazil, "where the only snail vector of *S. mansoni* is known as *A. nigricans*," found in two specimens "a complete pigmented fold running along the middle of the renal tube ... indistinguishable from the renal ridge found in *A. glabratus*." "In five other specimens the ridge was incomplete and the pigment scanty. In one specimen the pigment although not abundant was present while the ridge was lacking. In the remaining 43 specimens the renal region was smooth and no trace of any ridge or pigment could be detected. Cross-sections made through the renal ridge of one ridgy specimen (Fig. 1) showed that the structure found in the Niteroi's population has the same histological aspects already described by Paraense and Deslandes (1955) in *A. glabratus* from Belo Horizonte, State of Minas Gerais."

Further observations on *T. tenagophilus* by Barbosa *et al.*[2] gave the following results: 135 specimens from Niteroi: typical ridge (as in *T. glabratus*) in 8, incomplete ridge in 5, no ridge but only pigment in 1; 56 specimens from Macaé (Rio de Janeiro): well defined ridge in 26 (pigmented in 3, unpigmented in 23); 65 specimens from São Gonçalo (Rio de Janeiro): pigmented but incomplete ridge in 1; specimens from Magé and Terezopolis (Rio de Janeiro), Acari (Distrito Federal), Santos (São Paulo) and Belo Horizonte (Minas Gerais): renal surface smooth.

MATERIAL AND METHODS

This study is based on the examination of the renal region of 15,516 specimens of *T. glabratus*

* This study was aided by a grant from the Conselho Nacional de Pesquisas of Brazil.

† Following the Rules of Nomenclature[9] we use the generic name *Taphius* as the senior synonym of *Biomphalaria*, *Tropicorbis*, *Platytaphius*, *Afroplanorbis* and *Australorbis*. The species name *tenagophilus* refers to the large carinate Brazilian planorbid, until recently called *nigricans* by us; this nomenclature problem will be discussed elsewhere.

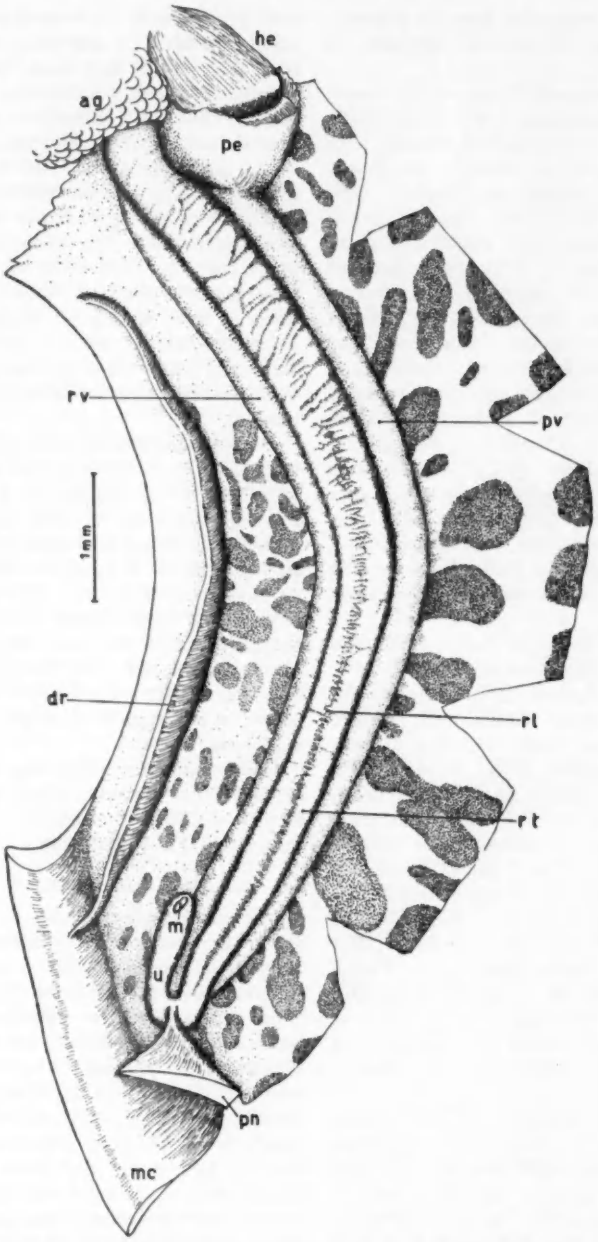


FIG. 1. Renal region of *T. glabratus* from Jacarezinho, 6 mm in shell diameter (ag, fragment of albumen gland; dr, dorsolateral ridge; he, heart; m, meatus of ureter; mc, mantle collar; pe, pericardium; pn, portion of pneumostome; pv, pulmonary vein; rl, renal pigmented line; rt, renal tube; rv, renal vein; u, ureter).

and 12,776 of *T. tenagophilus* from the following localities (numbers of examined specimens in parentheses):

T. glabratus. Venezuela: Caracas (38). Brazil: State of Pará: Capanema (190); State of Rio Grande do Norte: Lake of Estremoz (142), Lake of Papari (147); State of Paraiba: João Pessoa (82); State of Pernambuco: Paulista (45), Peixinhos (353), Recife (90); State of Bahia: Jequié (16), Salvador (89); State of Espirito Santo: Afonso Claudio (378), Baixo Guandú (293), Colatina (258), Itaguaçú (109), Itambé (28), Itarana (215), Mascarenhas (253), Santa Joana (100); State of Rio de Janeiro: Duas Barras (273); Distrito Federal: Manguinhos, population introduced from north-eastern Brazil in 1916 (500); State of Minas Gerais: Belo Horizonte (8,324), Conselheiro Pena (266), Governador Valadares (287), Jaboticatubas (60), Januaria (108), Jequitinhonha (83), Lagoa Santa (364), Mariana (50), Medina (93), Nova Era (80), Pedra Azul (184), Santa Luzia (935), Tarumirim (681); State of Goiás: Formosa (92); State of Paraná: Jacarezinho (188), Jataizinho (122).

T. tenagophilus. Brazil: State of Espirito Santo: Linhares (53); State of Rio de Janeiro: Cabo Frio (100), Campos (258), Cantagalo (5), Macaé (550), Niteroi (2,600), São Gonçalo (1,712), Tres Rios (116); Distrito Federal: Acari (42), Bonsucesso (148), Deodoro (20), Jacarepaguá (156), Quinta da Boa Vista (279); State of Minas Gerais: Belo Horizonte, three albino populations introduced with aquatic plants, probably from São Paulo (389), Itajubá (370), Juiz de Fora (85); State of São Paulo: Cubatão (19), Santo André (91), Santos (4,157), São Bernardo do Campo (362), São Paulo (380), São Vicente (118); State of Mato Grosso: Varzea Grande (100); State of Paraná: Curitiba (50), Jacarezinho (29), Paranaguá (187); State of Rio Grande do Sul: Guaiba (58), Porto Alegre (134), Tramandai (400). Argentina: Parana (37), Santa Fe (46).

For fixation and dissection, we followed the procedure described by Deslandes.[4] A specimen freely moving about, with the head and foot well exposed, is carefully picked up with a forceps, so as to prevent it from retracting back to the shell. The shell aperture is kept upwards, and the snail is gradually plunged in hot water at 60–70°C for about 30 seconds, the time varying with the animal's size. If the shell is plunged to the level of the aperture for about two-thirds of that time, the animal will relax satisfactorily and then, after being completely submerged, will die. Care must be taken not to exceed the suitable temperature and time of immersion, since blood and tissue cooking would hinder dissection. The dead animal is gently pulled by the foot with a small dentate forceps, so as to disconnect the insertions of the columella muscles. The whole animal is then drawn out of the shell, which remains unbroken. The animal is placed in Railliet-Henry's fluid, where it must remain for at least two hours before dissection. It could be left in the fixative for several years without damage, and dissection would still be well practicable after such a long time.

The animal is dissected under the stereoscopic microscope, partially immersed in the fixative to prevent it from drying. The fluid should not cover the specimen (to avoid fluctuation of it or its parts). Dissection begins by detaching the mantle along its left junction to the columella up to the level of the heart. The animal is then reversed, the connections of the right side of the mantle collar to the neck are sectioned, the mantle is separated from the right side of the body and finally cut off above the heart. Its inner (ventral) surface shows the renal tube and neighboring organs.

Histological observations were made on cross sections (12μ) of the detached mantle of numerous specimens, embedded in paraffin and stained with hematoxylin and eosin.

THE RENAL REGION IN *T. GLABRATUS*

The following organs are found on the ventral side of the mantle of *T. glabratus* (Fig. 1): 1. the pericardium (pe), posteriorly situated, enveloping the heart (he); 2. the kidney, consisting of an uppermost portion (the organ of Bojanus), connected to the pericardium, and of a descending tubular portion (the renal tube, rt), which bends anteriorly into a ureter (u) to empty by a subterminal meatus (m); 3. the pulmonary vein (pv), running along the right side of the renal tube, from the fore part of the mantle to the auricle of the heart; 4. the renal vein (rv), at the left of the renal tube, arising from the angle of the ureter; 5. the dorsolateral ridge (dr), a longitudinal fold on the left side of the mantle. From the taxonomic standpoint, the most important of these organs is the renal tube.

The mantle forms a pigmented background on which some darker stripes may be clearly seen. Along either side of the renal tube runs one of those stripes, marking the limit between the renal tube and the renal and pulmonary veins. Another stripe, less conspicuous, runs along the lateral side of each vein.

The aforementioned pigmented stripes are found even in very young specimens, less than 5 mm in shell diameter. As the animal grows, pigment gradually deposits on the ventral surface of the renal tube, or rather, in the cells of the extremely thin layer of connective tissue between the ventral wall of the renal tube and the epithelial lining of the mantle. Thus, in specimens of about 5 to 6 mm in diameter, the pigment, concentrated along the middle line, forms a dark stripe (Fig. 1, rl) first described by Pereira and Deslandes,[15] who regarded it as a forerunner of the renal ridge. The pigment concentration is especially remarkable in the distal half of the renal tube. It should be emphasized that this pigmented line *is strictly medial* and ends, anteriorly, near the bend of the ureter.

Fig. 7 is a cross section through the renal tube of the same specimen from Fig. 1. The mantle is covered dorsally with pigmented epithelium (de). The renal tube (rt), lined with cuboidal or columnar epithelium, lies between the pulmonary vein (pv) and the renal vein (rv), to which its lateral walls are juxtaposed. The ventral epithelium of the mantle (le) is cuboidal or columnar on the left and squamous on the right side. At the apex of the dorsolateral ridge (dr) it is ciliate and distinctly taller than in the surrounding surface. The change in the ventral epithelium, from columnar or cuboidal to squamous, takes place just under the renal tube. The vascular network of the mantle contains melanophorous cells in variable quantity, especially numerous in the following sites (shown in Fig. 7): a. the four angles between the lateral walls of the renal tube and the medial walls of the renal and pulmonary veins (these melanophores make up the pigmented stripes between the renal tube and the two veins); b. the lateral wall of each vein (these melanophores make up the lateral pigmented stripes of the veins); c. the framework of the dorsolateral ridge; d. the middle of the tissue layer between the renal tube and the ventral epithelium of the mantle (these melanophores make up the pigmented line that precedes the renal ridge).

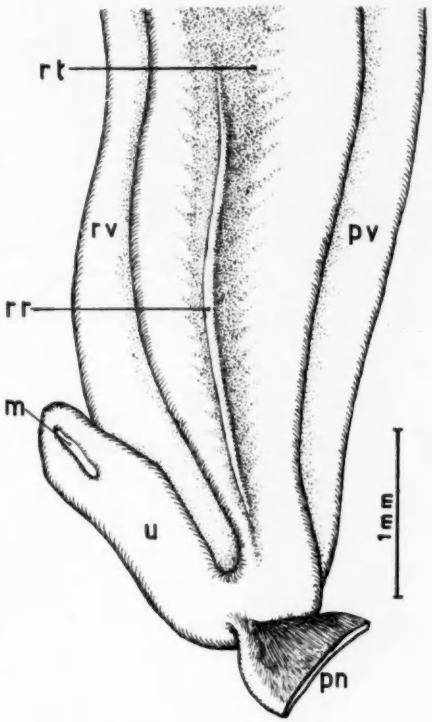


Fig. 2. Early stage of renal ridge in *T. glabratus* from Belo Horizonte, 10 mm in shell diameter (rr, renal ridge; other abbreviations as in Fig. 1).

The aforementioned melanophorous cells, for the most part, belong to the vascular network. Some of them, however, are rounded and oval amebocytes, either free in the blood spaces, or adherent to the walls of the latter. Owing to their role as defensive agents, the amebocytes may become extremely abundant in certain reactional processes (Fig. 13). In addition to the black granules of melanin, which in the strongly melanic animals may completely obscure the other cytoplasmic contents, all those cells also hold hemosiderin granules. The latter are comparatively large, ochreous in color, and especially visible in the albino specimens or in the less melanic ones. They easily give the Prussian blue reaction.

A careful microscopical examination of the renal region shows the existence of very slender longitudinal muscle fibers, just beneath the

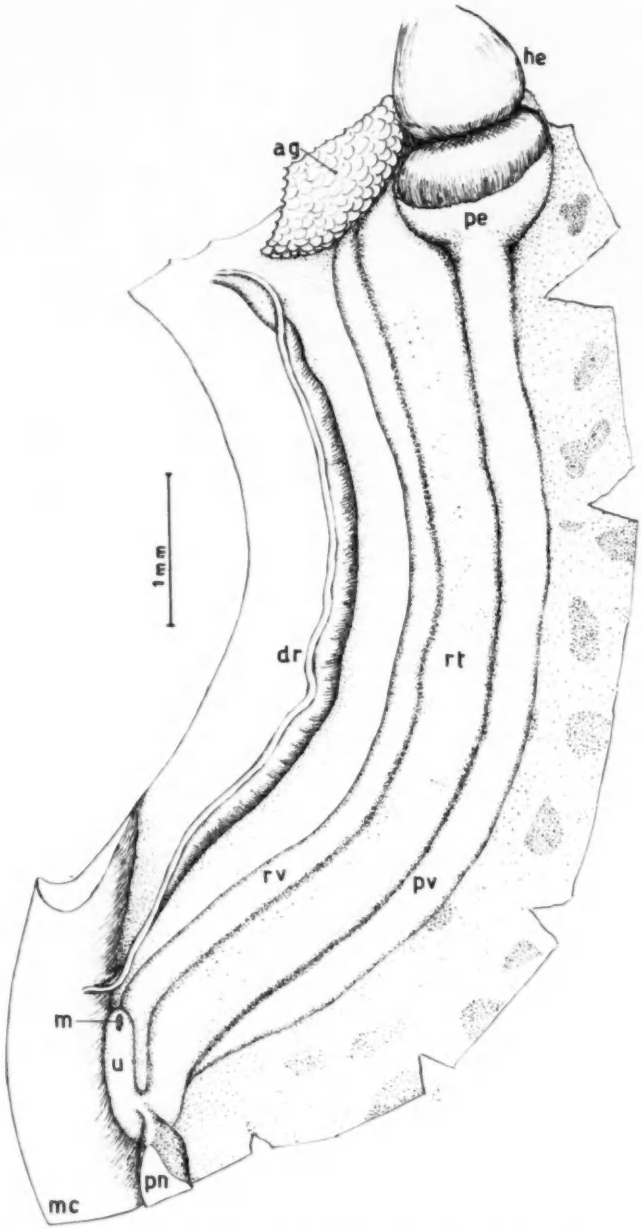
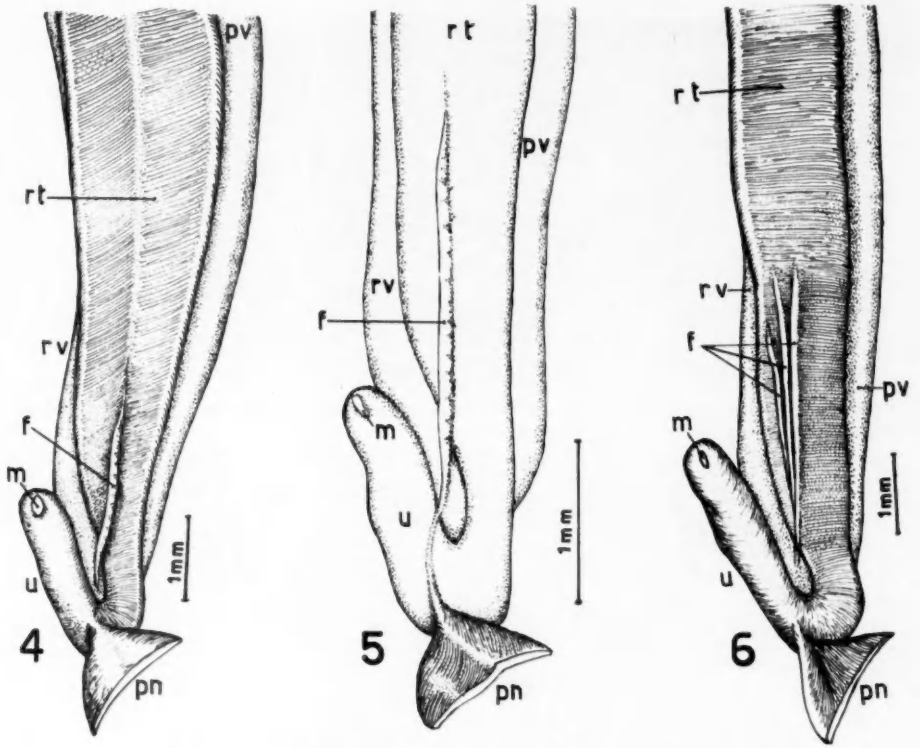


FIG. 3. Renal region of *T. tenagophilus* from Niteroi, 6 mm in shell diameter (abbreviations as in Fig. 1).



FIGS. 4, 5 and 6. False renal ridges (f,f,f) in *T. tenagophilus* from Macaé; specimens of 16, 15 and 17 mm in shell diameter, respectively (other abbreviations as in Fig. 1).

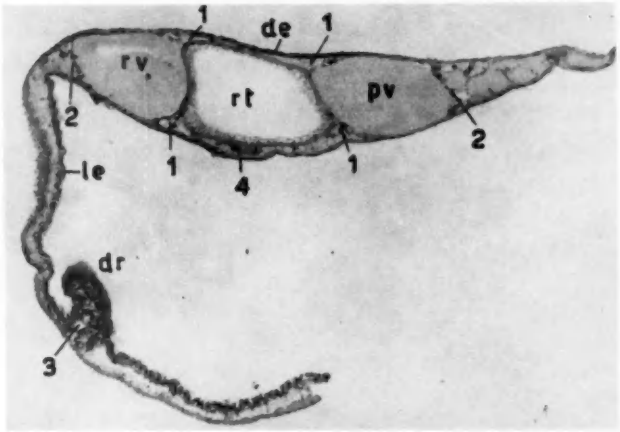


FIG. 7. Cross section through the renal region of *T. glabratus*, 6 mm in shell diameter, same specimen from Fig. 1 (de, dorsal epithelium of the mantle; dr, dorsolateral ridge; le, lining epithelium of the mantle; pv, pulmonary vein; rt, renal tube; rv, renal vein; 1, 2, 3, 4, sites where melanophorous cells are especially numerous). ×100.

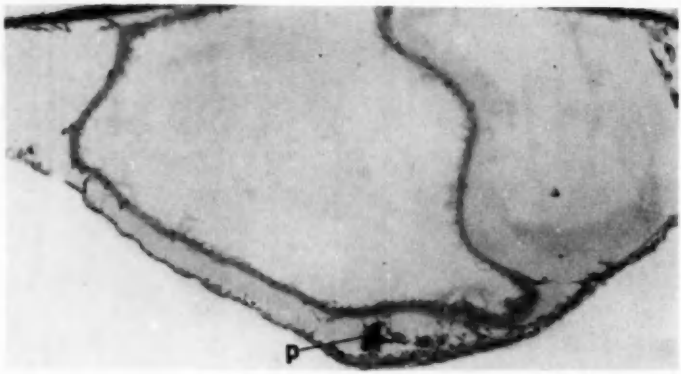


FIG. 8. Renal membrane of *T. glabratus* from Duas Barras, 9 mm in shell diameter, showing pigment (p) concentrated in the middle line. Cross section ×200.

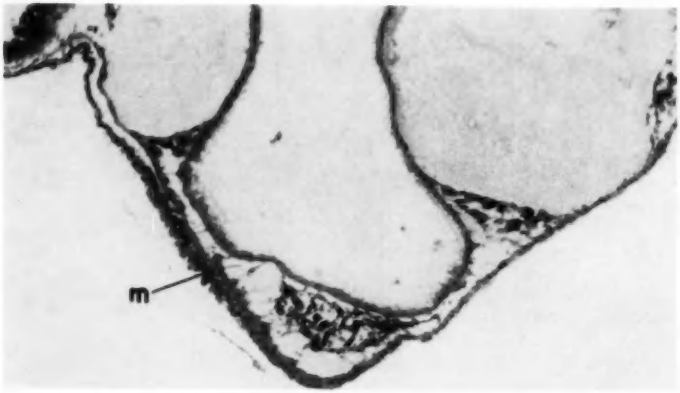


FIG. 9. Renal ridge of *T. glabratus* from João Pessoa, 12 mm in shell diameter, showing well developed subepithelial muscle (m). Cross section ×100.

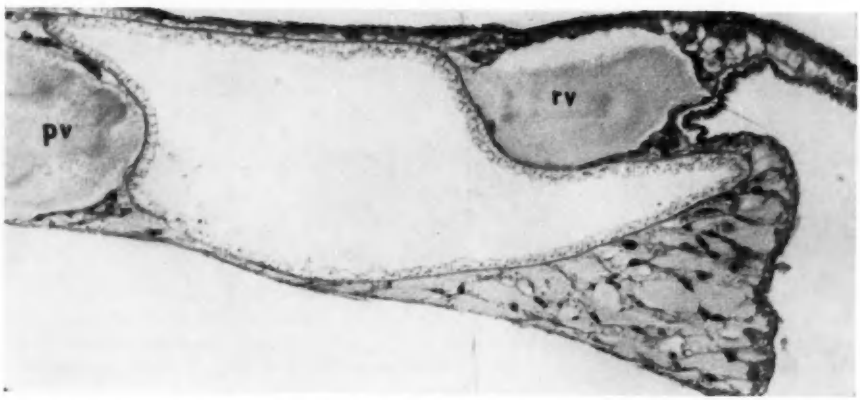


FIG. 10. Renal ridge (bent to the left) of *T. glabratus* from Peixinhos, 15 mm in shell diameter (pv, pulmonary vein; rv, renal vein). Cross section ×100.

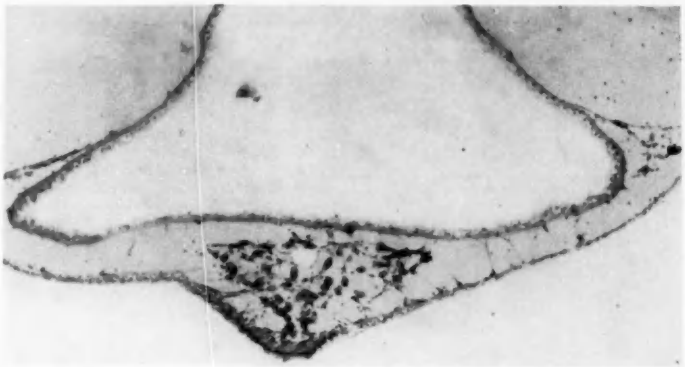


FIG. 11. Renal ridge of *T. glabratus* from Duas Barras, 18 mm in shell diameter. Cross section ×100.

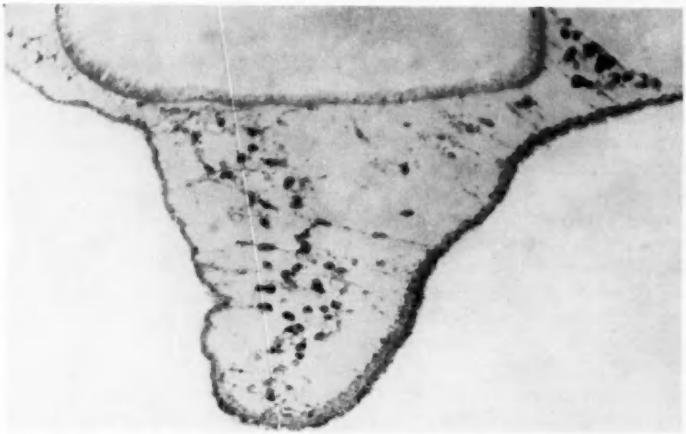


FIG. 12. Renal ridge of *T. glabratus* from Jacarezinho, 18 mm in shell diameter. Cross section ×100.

FIG. 13. Renal ridge of *T. glabratus* from Recife, 18 mm in shell diameter. Albino specimen showing reactional infiltrate of amebocytes. Cross section ×100.

FIG. 14. Cross section through the renal region of *T. glabratus* from Itarana, 24 mm in shell diameter, showing long renal ridge bent to the left. ×25.

epithelial lining of the mantle, especially concentrated along the middle line. These fibers are easily seen in the well developed renal ridge. At the distal end of the renal tube they join to the muscle fibers of the pneumostome.

In the growing animal there is a gradual increase, in size and number, of the blood spaces between the renal tube and the ventral epithelium of the mantle, with the result that the vascular tissue projects as a longitudinal ridge along the path of the medial pigmented line.[10] The earlier stage of the ridge is represented by a low permanent fold on the distal portion of the renal tube (Fig. 2). The development of that renal ridge is shown in Figures 7 to 14.

When well developed, the renal ridge consists of a vascular network, whose knots are occupied by stellate fixed melanophores, and shows an accumulation of the above mentioned amebocytes in its core. These cells, together with the fixed melanophores, give the ridge a melanic character. The ridge has a triangular shape in cross-section, with an apex pointing downwards and a base lying against the ventral wall of the renal tube. The outlines and dimensions of the triangle are widely variable, as it may be seen in sections of different specimens of the same size (Figs. 11 to 13).

The renal ridge, or the pigmented line preceding it, has been found in practically all the specimens of *T. glabratus*, over 3 mm in diameter, so far examined by us. The only exceptions were made by some small specimens from populations of less melanic individuals (*e.g.*, from Capanema, Medina and Jacarezinho), in which the pigmented line may appear somewhat later (in specimens of about 5 mm in diameter).

THE RENAL REGION IN *T. TENAGOPHILUS*

Except for the renal ridge and the pigmented line preceding it, the above description of the renal region of *T. glabratus* also holds for the closely related species, *T. tenagophilus*. A comparison between the two species was made by Paraense and Deslandes,[11] and further details are given now, with particular reference to the renal region.

As shown in Figure 3, which represents the renal region of a very young *T. tenagophilus*, 6 mm in shell diameter (to be compared with Fig. 1), the only significant difference, at this stage, between *T. tenagophilus* and *T. glabratus*, is the presence in the latter of the medial pigmented line. Another difference, of course liable to much individual variation, is in the pattern of pigmentation of the mantle. In both species, the pigment tends to concentrate into spots, but in *T. glabratus* the spots are darker and well outlined, whereas in *T. tenagophilus* they are paler and the intervening areas show some degree of diffuse pigmentation. In both cases, the spots tend to coalesce as the animal grows older. In *T. tenagophilus*, a complete coalescence is observed earlier than in *T. glabratus*.

Figure 15 is a cross-section through the renal tube of a small specimen, 9 mm in shell diameter. It may be seen that, contrary to what is observed in the small *T. glabratus*, the blood spaces of the ventral membrane show no tendency to form a network. Their walls are stretched vertically between the renal tube and the mantle epithelium, showing in cross-section a general palisade-like arrangement which persists in most specimens up to about 20 mm in diameter (Figs. 16 and 17). Only in larger specimens does a comparatively ill-developed network regu-

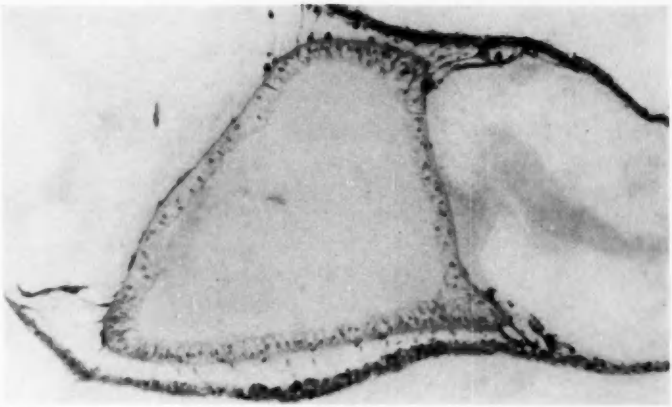


FIG. 15. Renal membrane of *T. tenagophilus* from Niteroi, 9 mm in shell diameter. Cross section ×200.

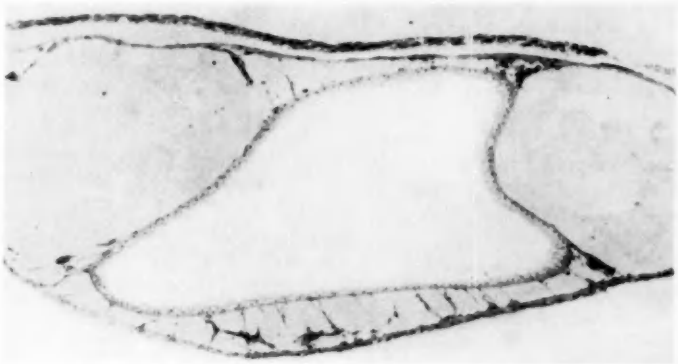


FIG. 16. Renal membrane of *T. tenagophilus* from Niteroi, 14 mm in shell diameter. Cross section ×100.

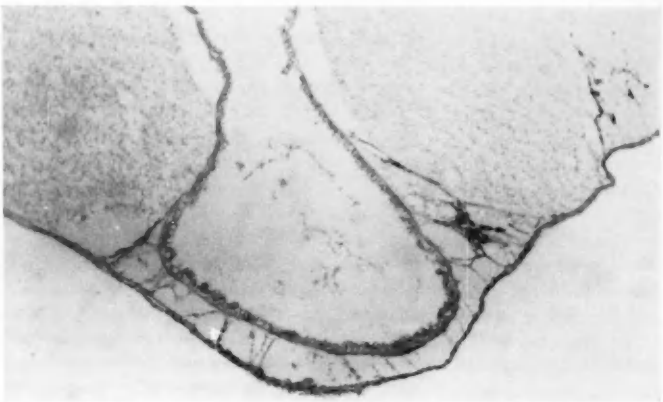


FIG. 17. Renal membrane of *T. tenagophilus* from Itajubá, 18 mm in shell diameter. Cross section ×100.

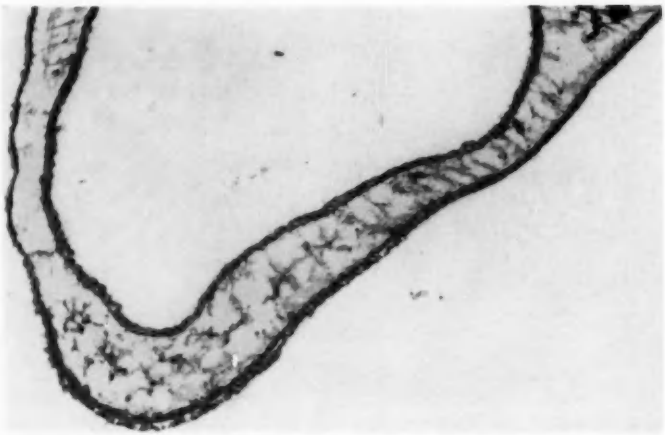


FIG. 18. Renal membrane of *T. tenagophilus* from Santo André, 21 mm in shell diameter. Cross section ×100.

larly show (Fig. 18). Another important difference is that, in each case, the ventral membrane of *T. tenagophilus* contains much less pigment than that of equally sized *T. glabratus*. This is due to a comparative paucity of fixed melanophores and to an almost complete absence of amebocytes in the ventral membrane of the former. When more abundant, the pigment cells are rather sparsely distributed, not concentrated in the middle line as in *T. glabratus*.

The difference in pigmentation of the renal membrane in the two species becomes more and more pronounced as the animals grow. Pigmentation, however, as a quantitative character, is liable to continuous variation and, therefore, the occurrence of variable degrees of pigmentation in the renal membrane of both species is to be expected. Whereas the individuals of *T. tenagophilus* are usually intensely melanic, their renal membrane is almost unpigmented in the vast majority of cases. In our abundant material, only exceptionally we have found something analogous to the medial pigmented stripe of the young *T. glabratus*, and even so in specimens over 13 mm in diameter. Even then, the pigmentation was not concentrated medially on the distal half of the renal membrane, as in *T. glabratus*, but followed a pattern similar to that shown in the proximal half in Figure 1. A cross-section of a specimen whose ventral membrane showed such a pigmented stripe is shown in Figure 16. In those instances no confusion with *T. glabratus* might be possible, seeing that, above 13 mm in diameter that species already shows a well developed renal ridge (Figs. 10 and 11).

VARIATION OF THE RENAL MEMBRANE IN *T. TENAGOPHILUS*

The above description of the renal membrane of *T. tenagophilus* refers to the typical specimens. In addition, a number of variants were found whose renal membrane showed unusual characteristics, some of which may be mistaken for a true ridge by some less experienced observer. Such variants are now considered separately, to show the differences between them and *T. glabratus*.

In some individuals, longitudinal wrinkles of variable length, already alluded to in another paper, may be found on the ventral membrane. A careful examination shows that they are due to fixation artifact.[11] Such artificial folds disappear if the ventral membrane is stretched transversally.

In other instances, the distal portion of the ventral membrane may show a low short fold, which bears some superficial resemblance to the early stage of the renal ridge of *T. glabratus*. This small fold, represented in Figures 4 to 6, was relatively frequent in the populations from Macaé, where it was found in 14.2 per cent of the 550 examined specimens. It is interesting to note that Barbosa *et al.*[2] record the presence of a well defined renal ridge in 26 of 56 specimens of *T. tenagophilus* from Macaé. In our present

investigation, however, nothing comparable to a true renal ridge was found. The aforementioned small fold occurred only in specimens over 14 mm in shell diameter, and showed no tendency to grow any further. At that size, the specimens of *T. glabratus* already show a definite ridge (Fig. 10) along the entire length of the renal tube. As shown in Figures 4 to 6, that small fold follows

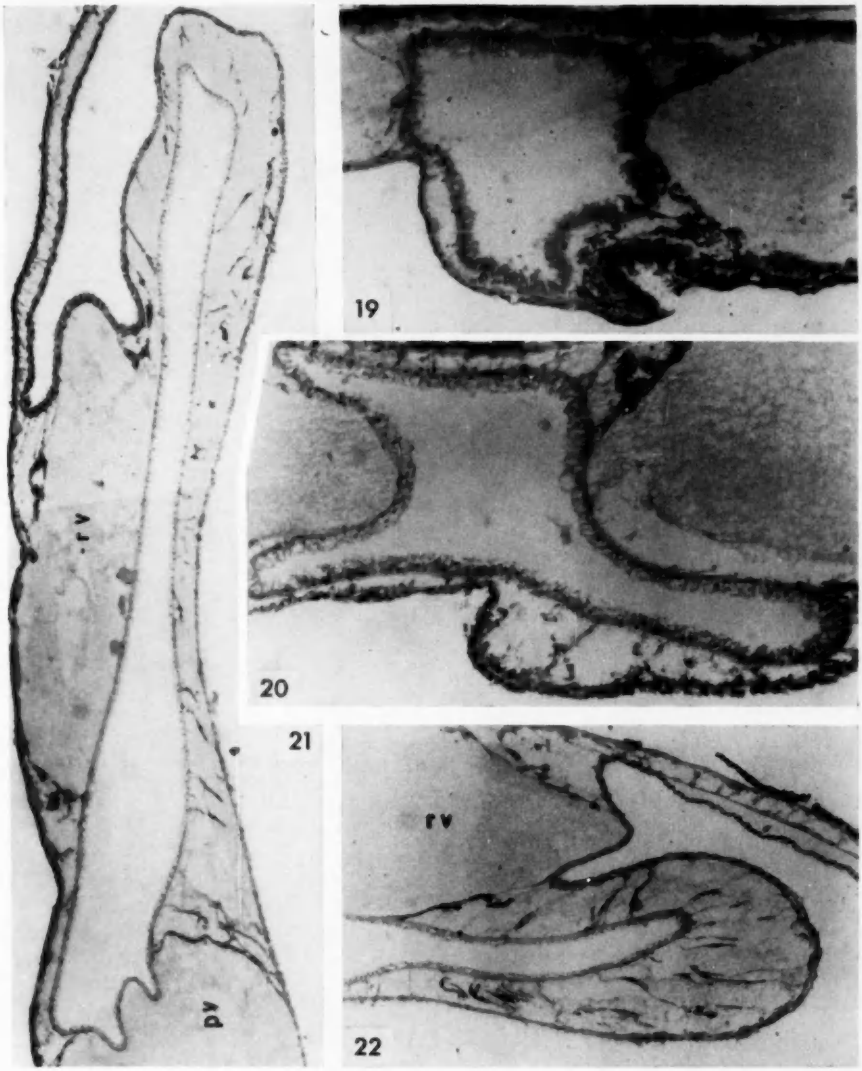


FIG. 19. False renal ridge of *T. tenagophilus*, same specimen from Fig. 5. Cross section near the ureter. ×200.

FIG. 20. The same specimen from Fig. 19, cross section at the middle of the fold. ×200.

FIG. 21. Renal membrane (bent to the left) of *T. tenagophilus* from Macaé, 22 mm in shell diameter (pv, pulmonary vein; rv, renal vein). Cross section ×100.

FIG. 22. Renal membrane (bent to the left and swollen at the free end) of *T. tenagophilus* from Niteroi, 23 mm in shell diameter (rv, renal vein). Cross section ×100.

an oblique course in relation to the renal tube, pointing to the space between the latter and the ureter. It differs from the early ridge of *T. glabratus* in that the latter is strictly medial in relation to the renal tube and points to the bend of the ureter (Fig. 2). That small fold seems to be caused by a rise of the terminal portion of the subepithelial muscle of the ventral membrane. As seen above, the fibers of that muscle connect to those of the pneumostome. Thus, the contraction of the latter may tighten the terminal portion of the subepithelial muscle and raise the corresponding portion of the membrane (Fig. 5). This interpretation seems to be backed by histological evidence. In cross-sections the fold appears as a mere rise of the epithelial lining under which the muscle fibers are always present, whereas the latter are missing in the unraised portion. Moreover, the subepithelial layer of vascular tissue shows a histological texture unlike that of a small true ridge (Figs. 19 and 20, compare Figs. 9 and 10).

In some specimens, especially in larger ones, the renal tube may lean to one side (most often to the left), thus partially or completely covering one of the lateral veins. In all those cases, no structure similar to a permanent ridge was observed, either by anatomical or by histological examination (Fig. 21). Such leaning of the renal tube is especially subject to be mistaken for a true ridge. In fact, the intersection of the two planes formed by the leaning membrane may sometimes look like a sharp angulation after fixation. By observing a crosscut slice under the dissecting microscope, it may be seen that the wall of the renal tube nearly accompanies the epithelial lining of the ventral membrane, instead of forming the base of a triangular ridge (compare Figs. 10 and 21). In some cases, the layer of vascular tissue somewhat swells at the free end of the leaning membrane (Fig. 22), but the texture of the thickened part does not differ from that of the typical membrane of *T. tenagophilus*. Such terminal swelling may stand

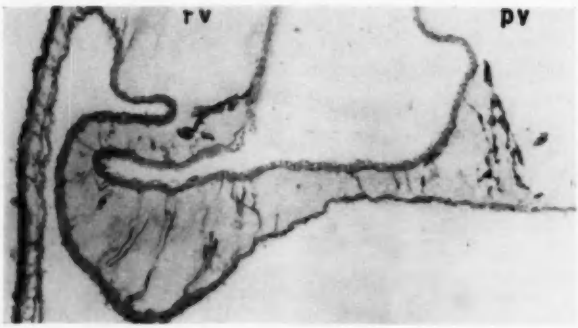


FIG. 23. Renal membrane (bent to the left and swollen ventrally at the free end) of *T. tenagophilus* from Macaé, 24 mm in shell diameter (pv, pulmonary vein; rv, renal vein). Cross section ×100.

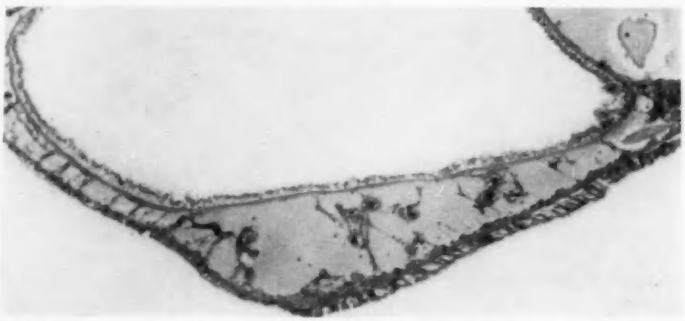


FIG. 24. Renal membrane (moderately swollen, showing widespread network) of *T. tenagophilus* from Macaé, 22 mm in shell diameter. Cross section ×100.

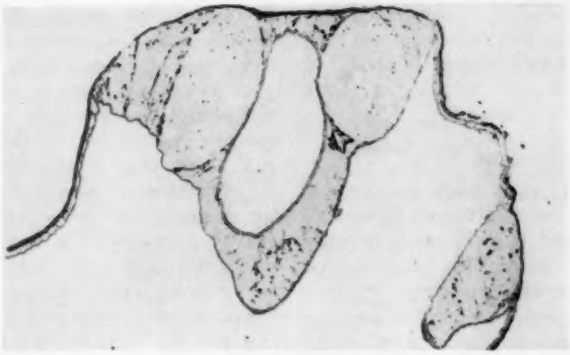


FIG. 25. Cross section through the renal region of *T. tenagophilus* from Santo André, 26 mm in shell diameter, showing abnormally swollen, diffusely reticulate renal membrane. ×25.

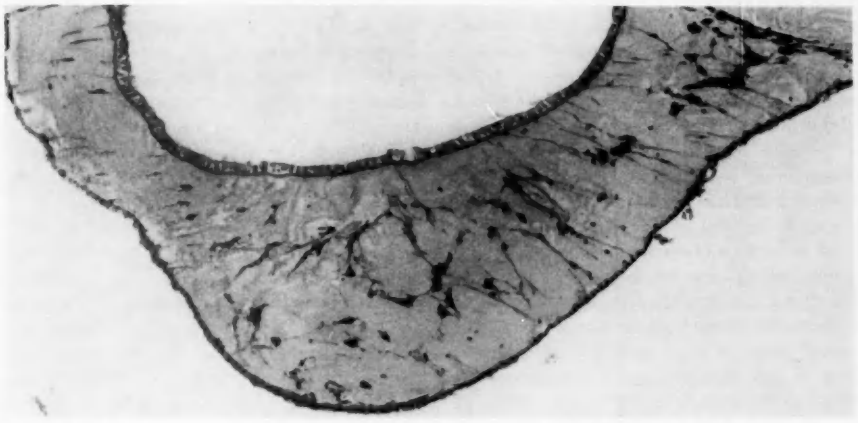


FIG. 26. Renal membrane (abnormally swollen and diffusely reticulate) of *T. tenagophilus* from Santo André, 25 mm in shell diameter. Cross section ×100.

ventrally (Fig. 23), simulating a ridge like that shown in Figure 10; again in this case the difference in texture of the vascular tissue is decisive.

In some of the larger specimens the ventral membrane may become much thicker than usual. Two extremely thickened membranes are shown in Figures 25 and 26. They belong to the two largest specimens of the sample from Santo André, which were 26 and 25 mm in diameter, respectively, and had typically wide, biconcave and bicarinate shells. The renal membrane of the largest specimen projected downward, looking like a blunt wide ridge. However, on account of the shell characters, and of the scarcity of pigment in the renal membrane, there was no doubt about the specific identity of this specimen. A cross section, examined under the dissecting microscope, showed that the ventral wall of the renal tube accompanied the projection of the ventral membrane, instead of forming the base of a triangular ridge as it would do in *T. glabratus*. Even disregarding the shell characters, no confusion with *T. glabratus* might be possible in this case, since even in specimens under 26 mm in diameter the renal ridge of that species is already an impressive structure (compare Figs. 14 and 25).

When abnormally swollen, the ventral membrane of the large specimens of *T. tenagophilus* tends to develop a vascular network similar to that normally present in *T. glabratus* (Figs. 24, 25 and 26). However, the histological examina-

tion (preferably of a series of sections) reveals that the network is rather widespread and only occasionally contains any amebocyte, whereas in *T. glabratus* it is confined to the ridge and shows at least a number of amebocytes.

DISCUSSION

In previous papers we gave a description, for systematic purpose, of the renal ridge of *T. glabratus*, and showed the differences between this species and *T. tenagophilus*, based on the characteristics of the renal region.[10, 11] Then we felt, however, that such a description was insufficient, and that a more elaborate one would be necessary to define clearly what we mean by "renal ridge" in *T. glabratus*.

In this paper the ridge is defined and our previous statements on its reliability as a diagnostic character of *T. glabratus* are confirmed. On the one hand, such a character makes it possible to separate this species from all the other Brazilian planorbids. On the other hand, it is the most consistent morphological feature that permits one to distinguish *T. glabratus* from the closely related *T. tenagophilus*.

According to Hubendick,[5] in *T. glabratus* "all stages from a well developed renal ridge to none at all can be observed. This vast range of variation seems, however, to be an exceptional rather than a common occurrence in planorbids." Our above mentioned observations show that the renal ridge of that species is a character subject to age variation, since it progressively develops as the animal grows. In this sense, we agree to Hubendick's statement. However, the variation of the character does not interfere with specific identification, seeing that, in the absence of the ridge, we have recourse to the pigmented line preceding it. The only serious difficulty lies on the young albino specimens, but such a difficulty has no practical significance owing to the rarity of albinos and to the possibility of examining larger specimens from the same population. Since age variation is a kind of progressive individual variation,[7] it is necessarily intrapopulational. So, the variation of the renal ridge in *T. glabratus* cannot be considered in terms of interpopulational variability, *i.e.*, it is highly doubtful that populations of this species, differing only in the presence or absence of renal ridge, may occur at all. If such difference is observed, the unridged population certainly belongs to another species, which in Brazil is represented by *T. tenagophilus*.

As seen above, there is no serious difficulty in distinguishing the true renal ridge of *T. glabratus* from the features that simulate a ridge in *T. tenagophilus*. In almost all doubtful cases, the examination of a mere crosscut slice under the dissecting microscope will suffice to clear away any doubt. The experienced observer readily sees that in the case of *T. tenagophilus* the triangular pigmented mass of vascular tissue is missing between the ventral wall of the renal tube and the lining epithelium of the mantle. Except for some exceptional case, the distinction can be made without resort to histological examination. The reference to histological sections in this paper is intended rather to complete our definition of the renal ridge than to give indispensable clues to the generality of cases. In this connection, we think that, if the beginner accustoms himself to examine all his doubtful cases histologically, he will gradually be able to do without this method and to recognize the diagnostic features at a mere inspection under the dissecting microscope.

In our material of *T. tenagophilus*, all the rare specimens that showed a pigmented stripe along the renal membrane had considerably exceeded the size at which that early character is present in *T. glabratus*. The same was observed in regard to the features simulating a renal ridge: they appeared in specimens of such a size that, to be confounded with *T. glabratus*, should have a true ridge much more developed than the false one actually shown. In the cases under discussion, the taxonomist will be much less liable to doubt if he realizes that the diagnostic characters of the renal membrane, being subject to age variation, must always be considered in association with the snail's size.

The results presented by Barbosa and Coelho[3] seem to contradict our foregoing statements. A careful examination of their paper, however, shows that they adduce no definite proof of the existence of a true renal ridge in *T. tenagophilus*. As mentioned in the introduction of the present study, they claim to have found two specimens with complete and five with incomplete ridge in 50 dissected specimens. Their illustration of a ridged kidney, however, does not belong to any

of those 50 dissected specimens, since it represents a section of a whole animal whose renal region had not been previously detached for anatomical examination. If that figure is representative of their ridged specimens, we can be sure that no true ridge occurred in their material. The figure in question bears a good deal of resemblance to our Figure 19, which is the cross section of a false ridge near the ureter. It should be noted that the somewhat greater length of their "ridge" may be explained by the obliquity of the section. The renal wall, as seen in really transverse sections, is lined by simple epithelium of uniform thickness. In the illustration under discussion, however, the ventral portion of the renal wall shows about five layers of nuclei, instead of a single one, and is five times as thick as the thinnest portion, on the upper left, where there is a single layer of cells. Owing to the obliquity of the section, the ventral membrane has the appearance of being longer than it would be if cut transversely.

So far, we have had no serious difficulty in recognizing either *T. glabratus* or *T. tenagophilus* on the basis of the shell characters associated with those of the renal region. Based on our extensive experience we can say, therefore, that if one should come upon any doubtful case, it would not invalidate the taxonomic significance and the practical value of the renal ridge.

Mandahl-Barth's[6] observations on the congeneric African species of *Biomphalaria* indicate that they may show a renal ridge, but that the latter occurs as mere "faint traces and in few specimens" (p. 1109). More detailed investigation on the nature of such ill developed and infrequent structure in the African species is desirable, since it may be in reality a false ridge like that sometimes occurring in *T. tenagophilus*.

SUMMARY

This paper is based on the comparative study of 15,516 specimens of *Taphius glabratus* from 36 localities and 12,776 of *Taphius tenagophilus* from 31 localities. A description of the renal region of both species is given, and it is concluded that they are distinguishable by the presence (in *T. glabratus*) or absence (in *T. tenagophilus*) of a ridge along the middle line of the membrane which covers ventrally the renal tube.

The ridge makes its appearance in specimens of about 10 mm in shell diameter and then gradually develops as the animal grows. It rises from a pigmented stripe which represents its forerunner in the small specimens under 10 mm in diameter.

The renal ridge is defined, both anatomically and histologically, and is shown to be a reliable diagnostic character of *T. glabratus* subject to age variation.

The unridged renal membrane of *T. tenagophilus* is also defined, with especial reference to its variation and to some unusual characteristics that may lead the taxonomist to confuse *T. tenagophilus* and *T. glabratus*.

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HOST-PARASITE RELATIONSHIPS IN ECHINOCOCCOSIS

III. Relation of Environmental Oxygen Tension to the Metabolism of Hydatid Scolices*

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Agosin *et al.*,[1] have demonstrated appreciable in vitro aerobic and anaerobic metabolic rates for the scolices of *Echinococcus granulosus*. It has not been shown, however, which of these two pathways predominates in the in vivo metabolism of hydatid tissues. In the present study, therefore, the oxygen consumption rate of hydatid scolices was determined as a function of the oxygen tension of the suspending medium. These data were then related to the in vivo oxygen tension of hydatid cyst fluid (HCF).

MATERIALS AND METHODS

Biological material. The tissues used in these experiments were scolices and brood capsules collected from bovine hydatid cysts obtained from the Beirut abattoir within two hours after slaughter of the host animals. Polarographic experiments were run within a few hours after the scolices were received. The suspending medium was HCF which had been filtered through paper to remove scolices and stored at −25°C until used.

Polarographic method. The instrument employed was a Sargent model XII recording polarograph with a modified H-type cell (Figure 1). The method for the polarographic determination of oxygen in biological fluids was essentially that of Petering and Daniels.[4] The cell, containing 25 ml of scolex suspension, was immersed in a constant temperature water bath at 37 ± 0.1°C. The suspension was frequently agitated with a sealed-in stirrer. The height of the mercury column was kept constant at 67.05 cm. The same capillary electrode was used throughout. The content of organic substances in HCF was sufficient to suppress the maximum of the first oxygen wave without the addition of gelatin. Polarograms were recorded on bromide papers and developed in Kodak D72. The tangent method[10] was used to determine the half-wave potentials.

Calibration. The polarograph was calibrated for oxygen by the ascorbic acid oxidase method.[8]

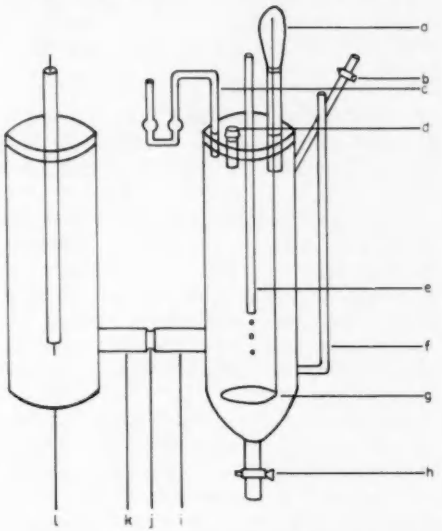


Fig. 1. Cell for polarographic determination of dissolved oxygen: (a) rubber balloon for manipulation of sealed-in stirrer, (b) sidearm and stop cock for introduction and removal of paraffin oil. Twenty-five ml of scolex suspension fill the cell to the level of this sidearm, (c) U-tube trap containing a few ml of paraffin oil to allow escape of excess gas during gassing of cell, (d) rubber diaphragm cap for introduction of scolex suspension or hydatid cyst fluid, (e) capillary dropping mercury electrode; the cathode, (f) capillary sidearm for gassing cell; sealed with paraffin wax after use, (g) sealed-in glass stirring rod, (h) stopcock for removal of mercury as it accumulates in the capillary reservoir, (i) sidearm packed with glass wool to prevent collection of scolices, (j) sintered glass disc, (k) agar bridge saturated with KCl at 25°C, (l) saturated calomel electrode; the anode.

* Aided by grants to the Department of Tropical Health from Pfizer International and the National Institute of Allergy and Infectious Diseases, U.S.P.H.S. (E-1769). Portion of a thesis submitted by the senior author for the degree of Master of Science in Chemistry.

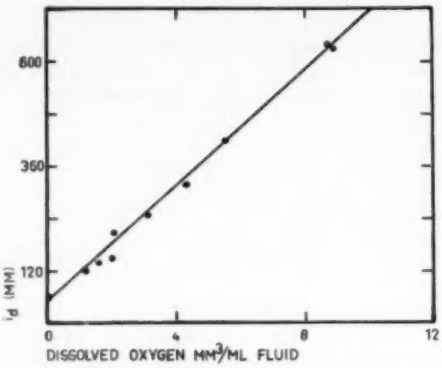


FIG. 2. Polarograph calibration curve for oxygen. Ascorbic acid oxidase method. Diffusion current (i_d) in mm against dissolved oxygen in mm³ per ml.

The enzyme was prepared from cucumbers. The calibration curve was repeated twice on 10 different samples of HCF (Figure 2). Calibration curves for dissolved oxygen in water were determined by the ascorbic acid oxidase method and by Winkler's method.[7] The two were practically identical. Winkler's method was not applicable, however, for oxygen determinations in HCF. Even when oxygen was bubbled through the fluid, Winkler values were invariably zero (possibly because of oxidizable organic material in the HCF).

Determination of in vivo oxygen tensions. Bovine lungs and livers infected with hydatid cysts were removed from the animals at slaughter and the HCF immediately drawn into sterile 50-ml glass syringes. A filter paper disk in a Swinny syringe-type filter prevented the introduction of scolices and brood capsules. The barrels and tips of the syringes were promptly sealed with Vaseline. Cysts from which fluids were withdrawn were examined for fertility. Fluids from nonfertile cysts were rejected. Syringes were quickly transported to the laboratory and the oxygen tension in the samples determined by the polarographic method. The sample was expelled into the polarographic cell by insertion of the syringe needle through the rubber diaphragm cap. The cells had been flushed previously with pure nitrogen.

RESULTS

Initial experiments showed that the small variations in electrolyte content and organic content among the different HCF used in these studies did not appreciably affect the half-wave potentials ($E^1_{\frac{1}{2}}$ and $E^2_{\frac{1}{2}}$) of oxygen, that no appreciable differences in i_d (diffusion current) could be noted with pH variations of 1 to 2 pH units and that sealing the cell with freshly boiled and cooled paraffin oil prevented appreciable changes in the oxygen concentration of filtered HCF under the conditions of the experiments (Table 1).

TABLE 1

Change in i_d with time of filtered hydatid cyst fluid in the polarograph cell with and without a paraffin oil seal

Time (*min*)	O_2, i_d (*mm*)		
	Without oil seal	With oil seal	
0	1020	960	125
10	1020	—	—
15	—	960	120
20	1020	—	—
30	980	—	115
35	—	—	120
40	900	—	—
45	—	940	—
60	880	940	—
70	900	—	—
75	—	—	115
80	800	—	—
90	800	880	115
105	—	—	120
210	—	860	—
Average rate of change (mm/min)	2.44	0.48	nil

In vivo oxygen content of hydatid cyst fluids. The dissolved oxygen content of the fluids of 10 hydatid cysts (6 to 10 cm in diameter) from two different bovine animals is shown in Table 2. The mean concentration in lung cysts was 2.91 ± 0.05* mm³ per ml and in liver cysts 1.60 ± 0.42 mm³ per ml.

Q_{o_2} of hydatid scolices as a function of O_2 tension. In six experiments, 25 ml of a suspension of scolices and brood capsules in HCF were introduced into the polarograph cell, which was then sealed with paraffin oil. Scolices from a 10-ml aliquot of the suspension were washed with water by suspension and centrifugation and dried to constant weight at 108°C. The dissolved oxygen

* Mean ± standard deviation.

TABLE 2

Dissolved oxygen content of hydatid cyst fluids

Animal number	Cyst number	Organ	Fluid pH	Diffusion current (i_d) (*mm*)	Oxygen content (*mm³/ml*)
1	1	liver	7.4	190	2.28
	2	liver	7.4	190	2.28
	3	lung	7.4	235	2.80
	4	lung	7.4	235	2.80
	5	lung	7.4	250	3.12
2	6	liver	7.4	125	1.36
	7	liver	7.4	120	1.28
	8	liver	7.4	120	1.28
	9	liver	7.4	120	1.28
	10	liver	7.4	130	1.44

TABLE 3

Run A of a typical experiment. Fluid not gassed. Dry weight of tissues: 306 mg

Time (*min*)	i_d (*mm*)	Fluid oxygen content (*mm³/ml*)	Q_{O_2} (*mm³/mg. dry wgt/hr*)
0	295	3.84	—
10	165	2.00	0.74
15	118	1.28	0.50
21	95	0.96	0.37
25	66	0.56	0.24
30	64	0.48	0
40	58	0.40	0
50	63	0.48	0
60	59	0.40	0
70	59	0.40	0

content of the HCF was then recorded each five to 10 minutes polarographically and these values converted to Q_{O_2} (mm³ of oxygen consumed per mg dry weight of tissue per hour). The curves obtained were reproducible and the results of the six experiments were in close agreement. The Q_{O_2} of scolices and brood capsules decreased with decreasing oxygen tension until at a tension of 0.80 ± 0.48 mm³ per ml† the oxygen consumption rate became and remained zero. The minimal oxygen tension obtained when HCF was bubbled with pure nitrogen was 0 mm³ per ml. The Q_{O_2} at an oxygen tension of 3.76 mm³ per ml, which represented the average oxygen tension of HCF saturated with air, varied from 0.39 to 1.50 mm³ oxygen consumed per mg dry weight of tissue per hour (0.85 ± 0.39) in the 6 experiments. This compared with average manometric Q_{O_2}'s in HCF of 2.16[1] and 1.68 (Schwabe, unpublished data). The Q_{O_2} obtained at the average in vivo oxygen tension of hydatid cysts of the lung was approximately 96 per cent, while that at the average in vivo oxygen tension of liver cysts was approximately 54 per cent of this "optimal rate" for each experiment.

Detailed results of a single experiment. The change in environmental oxygen tension with time and in Q_{O_2} of scolices with decreasing oxygen tension is shown in Table 3 and Figures 3 and 5 (Curves A). The dry weight of scolices in the 25-ml suspension was 306 mg. Following run A the suspension was bubbled with oxygen to a concentration of 9.4 mm³ per ml and the readings repeated (Figures 3 and 5, Curves B). The

† For 5 of the 6 experiments this value was 0.56 ± 0.13.

TABLE 4

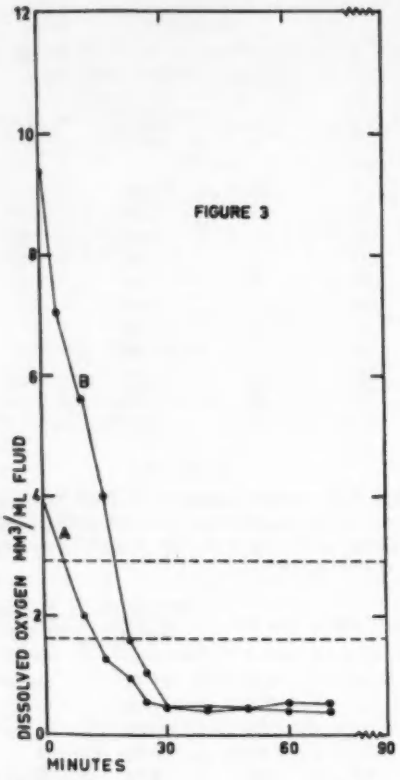
Run E of a typical experiment. Held 8 hours in refrigerator (oxygen content 0.50 mm³/ml), then gassed with oxygen to 11.84 mm³/ml. Dry weight of tissues: 306 mg

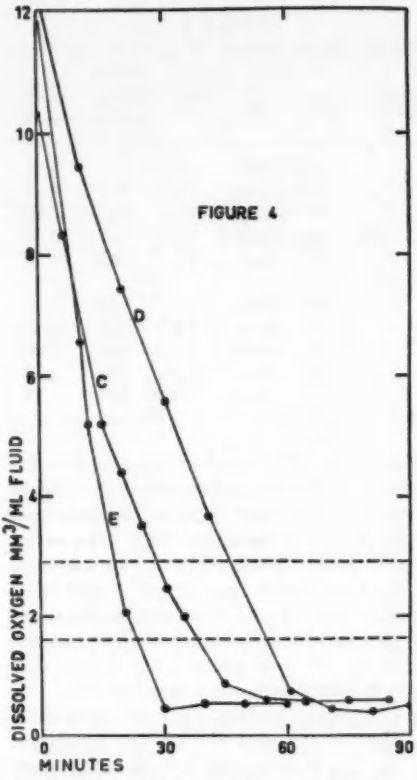
Time (*min*)	i_d (*mm*)	Fluid oxygen content (*mm³/ml*)	Q_{O_2} (*mm³/mg dry wgt/hr*)
0	850	11.84	2.60*
12	390	5.20	2.06
21	175	2.08	1.39
30	64	0.48	0
40	66	0.56	0
50	66	0.56	0
60	66	0.56	0

* Calculated at 9.68 mm³/ml.

suspension was rebubbled with oxygen a second and a third time to concentrations of 10.3 and 12.2 mm³ per ml, respectively, and additional readings recorded (Figures 4 and 5, Curves C and D). After run D the cell was placed in an air tight container and stored in a refrigerator for 8 hours. At the end of this period the oxygen concentration of the HCF was still 0.5 mm³ per ml. The cell was rebubbled with oxygen to a level of 11.8 mm³ per ml and readings again recorded (Figures 4 and 5, Curve E). Oxygen consumption rates on run E were greater than those obtained on the previous runs (Figure 5). Finally, the scolices were examined microscopically and were found to be invaginated, motile and otherwise normal in appearance.

Several runs were made in each of the experiments to confirm the continued viability of the





FIGS. 3 and 4. Decrease in oxygen content of hydatid cyst fluid with time as the result of scolex respiration. Five successive runs. Dry weight of tissue: 306 mg. Broken lines indicate in vivo oxygen content of fluids from hydatid cysts of lungs (above) and liver (below). Data from a typical experiment.

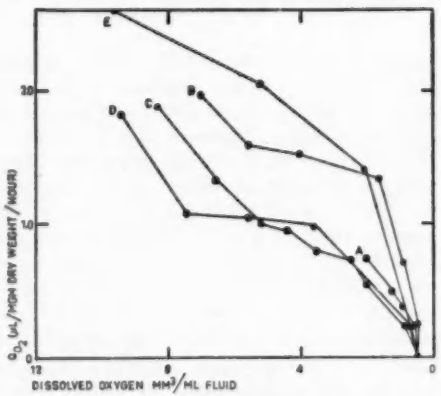


FIG. 5. Q_{O_2} (mm³ oxygen consumed per mg dry weight of tissue per hour) of hydatid scolices as a function of the oxygen content of the suspending medium. Five successive runs. Data from a typical experiment.

tissues and the absence of mercury toxicity under the experimental conditions (mercury was drawn off from the small pool in the bottom of the cell as it accumulated). The increased Q_{O_2} observed following anaerobic incubation suggested the repayment of an accumulated oxygen debt. A similar increase in Q_{O_2} was noted in the one other experiment in which the suspension was held anaerobically between runs.

DISCUSSION

The scolices and brood capsules of *Echinococcus granulosus* are attached to the germinal membrane and suspended freely throughout the hydatid cyst fluid. Agosin *et al.*[1] have demonstrated manometrically that these tissues respire in vitro at an appreciable rate in solutions at equilibrium with air. This observation has been confirmed by work in our laboratory (Schwabe, unpublished data). Earlier it had

been suggested by von Brand[9] that hydatid tissues lived in an oxygen poor environment and were probably dependent in vivo upon anaerobic pathways. Rogers[5, 6] had shown, however, that even at the low oxygen tensions which prevailed along the gut mucosa in rats and sheep, certain small nematodes were able to respire at from 40 to 80 per cent of their "maximum in vitro rates." He defined "maximum in vitro rate" as the Q_{O_2} of worms as determined by the direct method of Warburg in a gas phase of air. We have compared our polarographic rates on the same basis but prefer to refer to Rogers's rate as the "optimum in vitro rate" since higher Q_{O_2}'s were obtained at oxygen tensions greater than that of air.

The results reported here demonstrate that, at the in vivo oxygen tensions of HCF, hydatid tissues are able to respire at from 54 to 96 per cent of their "optimum rate." It would appear, therefore, that hydatid tissues normally metabolize aerobically in vivo, although they are possibly able to survive for considerable periods of time in the absence of oxygen. In a few preliminary Thunberg experiments (Schwabe, unpublished data), it has been observed that intact scolices and scolex homogenates, but not filtered HCF, possess an appreciable endogenous dehydrogenase activity.

It is of interest to note that the oxygen tension of HCF is greater in lung cysts than in liver cysts. Previously de Waele[2] had reported that HCF is free from oxygen. It is possible that his method ("pyrogallic acid test"), like the Winkler method, may have been unsuitable for oxygen determinations in a complex organic mixture such as hydatid cyst fluid.

The polarographic method for oxygen is well suited to experiments on aerobiasis and anaerobiasis. So far as we are aware, it has not been applied previously to studies on parasite physiology. It has distinct advantages over both the manometric method of Rogers[6] and the Cartesian diver method of Hunter and Vernberg:[3] (a) It is simpler to perform than the latter methods, both of which require the preparation of gas mixtures, (b) the Q_{O_2} can easily be determined at any number of oxygen tensions and (c) results are quickly obtained in single experiments employing the *same* tissues, whereas different batches of tissue and separate runs are required with the other methods.

SUMMARY

The polarographic method for oxygen was applied to the problem of aerobiasis-anaerobiasis in echinococcosis to show that:

1. The dissolved oxygen content of fluids of 3 hydatid cysts of the bovine lung ranged from 2.80 to 3.12 mm³ per ml, of 7 hydatid cysts of the bovine liver from 1.28 to 2.28 mm³ per ml.

2. The Q_{O_2} of hydatid scolices decreased with environmental oxygen tension until at a tension of 0.80 ± 0.48 mm³ per ml the rate became and remained zero.

3. The polarographic Q_{O_2} at an oxygen tension representing hydatid cyst fluid saturated with air ("optimum rate") ranged from 0.39 to 1.50 (0.85 ± 0.39).

4. The Q_{O_2} at the in vivo oxygen tension of lung cysts was 96 per cent and of liver cysts 54 per cent of the mean "optimum rate." It would appear therefore that hydatid scolices are able to carry on predominantly aerobic metabolism in vivo.

5. Hydatid scolices possibly repay an accumulated oxygen debt following anaerobic incubation.

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EPIDEMIC JUNGLE FEVER AMONG OKINAWAN COLONISTS IN THE BOLIVIAN RAIN FOREST

II. Isolation and Characterization of Uruma Virus, A Newly Recognized Human Pathogen

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During August and September 1954, 400 Okinawans were relocated to Bolivia under the auspices of the Bolivian and Okinawan Governments and the U. S. Department of State. After a long sea voyage and difficult overland travel through Brazil, the immigrants settled under extremely primitive conditions on a tract of cleared rain forest, called Uruma colony, 60 kilometers northeast of Santa Cruz, Bolivia. Starting in early October 1954, and continuing through the following spring, a febrile disease of nonspecific symptomatology swept through the colony, affecting 192 of the colonists and resulting in 15 deaths. An account of the clinical and epidemiologic features of the outbreak and attempts to determine its cause constitutes the first paper of this series.[8] Subsequent to the work reported therein, a previously unrecognized virus was recovered from the blood of one patient. This paper presents information concerning the isolation and characterization of the new agent, called Uruma virus, and preliminary data on its relationship to other viruses.

VIRUS ISOLATION

During the visit to Uruma colony of the medical field team assigned to investigate the outbreak in March 1955, blood specimens from three patients who had been febrile for 24 to 48 hours were inoculated into adult mice, guinea pigs and rabbits by the intracerebral and intraperitoneal routes, and into cultures of HeLa and MBIII cells. Precarious travel conditions resulted in death of the mice and guinea pigs a few days following inoculation, and passage of their tissues did not yield a transmissible agent. Rabbits so inoculated remained well for 30 days. The inoculated cell cultures were maintained in the field without incubation, refed twice with nutrient medium, and returned to the Communicable Disease Center (CDC) 10 days after inoculation. Upon receipt of the cultures at the CDC, fluids from each of the cultures were subinoculated intracerebrally into 1- to 2-day-old mice and into freshly prepared cell cultures. A transmissible agent, lethal for suckling mice, was recovered from an MBIII cell culture inoculated with the fresh blood of one patient. No cellular degeneration attributable to the agent was observed in the culture used for primary isolation or in that employed for subsequent passage. However, the agent propagated readily in 1-day-old-mice, killing early passage animals within 4 to 7 days after inoculation. By the fourth passage, the survival time of suckling mice inoculated intracerebrally with a 10 per cent suspension of infected brain was reduced to 2 to 4 days. At this passage level, infected brain suspensions contained 10^8 to 10^9 LD_{50} of virus per ml. Further characterization of the agent was carried out at the Walter Reed Army Institute of Research on virus suspensions derived from the 4th to 7th mouse passages.

Subsequent to its recovery from the MBIII cell culture, the agent was isolated from frozen

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TABLE 1

Gradocol filtration of Uruma virus

APD* of Gradocol membrane (mμ)	Infectivity titer of unfiltered virus preparation	Infectivity titer of filtrate	Log reduction in infectivity
220	$10^{-6.8}$	$10^{-4.5}$	1.3
160	$10^{-6.8}$	$10^{-3.8}$	3.0
110	$10^{-6.6}$	$10^{-3.3}$	3.3
95	$10^{-6.6}$	$10^{-1.6}$	4.0
79	$10^{-6.3}$	$10^{-1.6}$	4.7
52	$10^{-6.3}$	$<10^{-0.5}$	>5.7
31	$10^{-5.5}$	$<10^{-0.5}$	>5.0
95	$10^{-5.9}$	$10^{-3.6}$	2.3
79	$10^{-6.2}$	$10^{-2.0}$	4.2
52	$10^{-5.7}$	$<10^{-0.5}$	>5.2

* APD = average pore diameter.

serum and blood clot of the same patient by direct intracerebral inoculation of 1-day-old mice. The virus was later also similarly recovered from the stored frozen blood clot of another of the three patients seen in the acute stage of illness in March 1955.

PRELIMINARY CHARACTERIZATION

The viral nature of the recovered agent was established by its filtrability and its failure to propagate in cell free media. Thus, the agent readily passed through bacteria tight Seitz EK and Selas-type filters and failed to proliferate on blood agar, thioglycollate broth, Difco PPLO medium, and Fletcher's and Schüffner's leptospiral culture media.

The approximate size of Uruma virus was determined by filtration, using Gradocol membranes of graded pore sizes. Prior to filtration the supernate (RCF = 1800 G for 15 minutes) from a 10 per cent infected mouse brain suspension was precipitated with powdered protamine sulfate (5 mg per ml) to remove the majority of tissue debris.[9] The virus-containing supernate from this precipitation was in turn passed through an ultrafine sintered glass filter. The resulting filtrate served as starting material for filtrations through membranes of successively diminishing pore diameters. The results of two experiments are presented in Table 1. In both experiments, virus passed through membranes with an average pore diameter (APD) of 79 mμ, but was retained by membranes with an APD of 52 mμ. Applying the Elford correction factor[6] to the filtration end-points, the particle size of Uruma virus was estimated to be 17 to 26 mμ. Preliminary ultracentrifugation data have been in accord with the filtration results and indicate a particle size of about 25 mμ.

TABLE 2

Stability of Uruma virus at 56°C*

Heating interval (*min*)	Log infectivity titer after heating at 56°C
Unheated	8.5
10	3.8
20	3.0
30	<0.5
45	<0.5
60	<0.5

* Tested as 10% suspension of infected mouse brain in phosphate saline (pH 7.6) after low speed centrifugation (RCF 1800 G/15 min).

That the viability of Uruma virus is relatively stable is shown by its isolation from a cell culture and blood specimens which were maintained without refrigeration in the field for several days and by a minimal loss (less than 0.5 log) of infectivity after storage at −70°C for 2 years. Lyophilized suspensions of infected brain tissue in 0.02 M phosphate-buffered saline (pH 7.6) containing 50 per cent normal rabbit serum have yielded viable virus after a similar period of storage at 4°C. Further information on the heat stability of Uruma virus was obtained by titrating viral suspensions after heating at 56°C for varying time intervals (Table 2). The infectivity of virus was destroyed after 30 minutes at 56°C.

HOST RANGE

Data on the pathogenicity of Uruma virus for various experimental hosts are summarized in Table 3. One-day-old mice are highly susceptible to infection with Uruma virus when inoculated intracerebrally or intraperitoneally. Mouse susceptibility decreases markedly with an increase in age; thus, 3- to 4-day-old mice were more refractory to infection than 1-day-old animals, as indicated by a lower infectivity titer and a longer survival time. Ten-day-old mice uniformly survived intracerebral inoculation of 10^7 infant mouse LD_{50} of virus.

Guinea pigs inoculated intracerebrally with 10^7 mouse LD_{50} occasionally developed fever after 4 to 6 days, but usually survived. Serial

TABLE 3

Pathogenicity of Uruma virus for various experimental hosts

Host	Inoculum		Reaction	Remarks
	LD_{50}*	Route		
Animals:				
Swiss mouse (Bagg strain)	10^1–10^8	i.c.	Paralysis, death	Survival time—2–6 days
	10^1–10^8	i.c.	Paralysis, death	Survival time—2–6 days
1 day old	10^1	i.c.	Paralysis, death of 7/9	Avg. survival time—3.4 days
	10^2	i.c.	Paralysis, death of 8/8	Avg. survival time—3.2 days
3–4 days old	10^1	i.c.	Death of 0/7	
	10^2	i.c.	Death of 1/9	Survival time—5 days
	10^3	i.c.	Death of 5/8	Avg. survival time—5.2 days
10 days old	10^4	i.c.	7/7 survived without symptoms	
	10^5	i.c.	11/11 survived without symptoms	
	10^6	i.c.	6/6 survived without symptoms	
	10^7	i.c.	8/8 survived without symptoms	
Guinea pig (400 gm)	10^7	i.c.	Fever in 4/12	
	10^7	i.p.	Death of 2/12 Fever in 1/4; survived	Virus recovered from brain; serial passage unsuccessful
Hamster				
1–3 days old	10^2	i.p.	Paralysis, death of 5/6	Avg. survival time—3.5 days
6 weeks old	10^7	i.c.	Occasional deaths	Serial passage unsuccessful
Rabbit				
(2–2.5 kg)	5×10^7	i.p.	Survived without symptoms	
Embryonated hen's eggs:				
(7–8 days old)	10^7	yolk sac	Death of embryo	Survival time = 3–7 days: 7 serial passages successful
	10^5	yolk sac	Occasional embryo deaths	
Tissue cultures:				
Hamster kidney	10^7		Complete cellular destruction in 3–4 days	Supernate from 2nd passage titered $10^{-4.7}$ in suckling mice
KB human carcinoma (Eagle)	10^4–10^6		Complete cellular destruction in 4–6 days	Supernate from 3rd passage titered $10^{-4.2}$ in suckling mice
Human liver (Chang)	10^7		Complete cellular destruction in 5–9 days	Serial passage successful

TABLE 3—(*Continued*)

Host	Inoculum		Reaction	Remarks
	LD_{50}*	Route		
Monkey kidney	10^7		Complete cellular destruction in 8–9 days	
HeLa	10^7		No cytopathogenic effect	
MBIII Mouse lymphosarcoma	10^7		No cytopathogenic effect	
Human conjunctiva (Chang 101)	10^7		No cytopathogenic effect	

* Based on intracerebral titrations in 1-day-old mice.

passage of the virus in guinea pigs was unsuccessful.

Although newborn hamsters, like mice, were highly susceptible, adult hamsters, like guinea pigs, survived following inoculation of massive doses of virus; only occasional deaths were observed following intracerebral and intraperitoneal inoculation of a 10 per cent virus suspension.

Young adult rabbits remained well following intraperitoneal inoculation of 5×10^7 infant mouse LD_{50} of virus, but responded to inoculation by producing significant amounts of neutralizing antibody.

Seven- to 8-day old embryonated hen's eggs proved susceptible to Uruma virus infection when inoculated into the yolk sac. By the 7th egg passage, inoculation of a 10 per cent virus suspension consistently caused death of embryos after an average survival time of 3.6 days. Yolk sac inoculation of 10^{-2} and 10^{-3} dilutions of virus did not result in a 100 per cent mortality rate.

Of seven cell lines tested for their ability to support growth of Uruma virus, four were susceptible to infection. In order of decreasing susceptibility, as judged by the time of appearance of cellular degeneration, these were: hamster kidney, KB human carcinoma (Eagle), human liver (Chang) and monkey kidney cells. Complete cellular destruction was observed in cultures of these cells in 3 to 4, 4 to 6, 5 to 9 and 8 to 9 days after inoculation, respectively. No cytopathic changes were observed in cultures of HeLa, human conjunctiva (Chang) or MBIII cells followed for 10 to 12 days after inoculation. The possibility of viral proliferation in the absence of cellular degeneration in the latter three cell types was not investigated. We are grateful to Miss Elizabeth B. Jackson for her assistance in chick embryo and tissue culture passage of the virus.

IMMUNOLOGIC CLASSIFICATION

Neutralization tests. Virus-dilution neutralization tests were utilized in initial attempts to identify Uruma virus by immunologic means. Hyperimmune antisera to the following viruses, though capable of neutralizing from 100 to 10,000 LD_{50} of homologous virus, failed to neutralize Uruma virus: Japanese encephalitis, Murray Valley encephalitis, West Nile fever, St. Louis encephalitis, dengue (type 1 and 2), yellow fever, Ntaya, Zika, Russian spring-summer encephalitis, Ilheus, Uganda S, Eastern equine encephalitis, Western equine encephalitis, Venezuelan equine encephalitis, Wyeomyia, Phlebotomus fever (Naples and Sicily strains), Anopheles A, Anopheles B, California, Colorado tick fever, Bunyamwera, herpes simplex, mouse encephalomyelitis (GDVII), encephalomyocarditis, lymphocytic choriomeningitis, Coxsackie A (types 1 and 2) and Coxsackie B (types 1 and 2). Uruma virus was neutralized by its homologous antiserum (neutralization index 5000) as well as by Semliki Forest and Sindbis hyperimmune sera. In three tests in which sera to the latter two agents were tested against Uruma virus, Semliki Forest antiserum neutralized 108, 32 and 25 LD_{50}, and Sindbis antiserum neutralized 25, 32 and 8 LD_{50} of Uruma virus. Neutralization of such small amounts of virus by heterologous sera would not have been considered insignificant were it not for repeated confirmation of the findings with Semliki Forest serum in different tests and the complete failure of other heterologous sera to neutralize as little as 3

LD_{50} of Uruma virus. Sindbis antisera continued to give results in the equivocal range of significance. The neutralization test data indicated that Uruma virus is distinct from at least 32 of the known neurotropic viruses, but that it is antigenically related to Semliki Forest virus and to a lesser extent, to Sindbis virus.

After demonstrating a relationship of Uruma to Semliki Forest and Sindbis viruses, information was received from Causey[4] concerning a virus (H290) isolated from a febrile worker clearing forest near Belem, Brazil. This virus exhibited a host range similar to that of Uruma virus and also appeared to be immunologically related to Semliki Forest virus. To determine whether the Uruma and H290 viruses were related, acute and convalescent sera from the patient from whom H290 virus was isolated were tested for neutralizing antibody to Uruma virus. The neutralization indices of the sera were found to be 0 and 2000, respectively, suggesting a close antigenic relationship between the two viruses. Subsequent work on H290 virus has shown it to be closely related or identical to Mayaro virus, as judged by hemagglutination-inhibition tests.[3] A more extensive study of serologic cross-reactions within the Group A arthropod borne viruses, using rabbit antisera, has been carried out by Dr. Edward Buescher of the Walter Reed Army Institute of Research. His results confirm the serologic differences between Mayaro and Uruma viruses. Further evidence for the relationship between Uruma, Mayaro, Semliki Forest and other viruses was provided by hemagglutination and complement-fixation studies.

Hemagglutination (HA) and hemagglutination-inhibition (HI) studies. Casals[1] has shown that many of the neurotropic viruses may be classified into one of three groups on the basis of HA and HI tests. Thus, an unknown isolate may be grouped by determining: (a) the optimum pH and temperature for agglutination of chick erythrocytes by the viral antigen, and (b) the extent of HI exerted by antisera to known viruses from each of the groups. This procedure was applied to Uruma virus following techniques employed in the Rockefeller Foundation Virus Laboratories.[7]

Attempts were made to prepare HA antigen from two sources: infected suckling mouse brain harvested 3 days after intracerebral inoculation with 10^6 LD_{50}, and serum of moribund mice drawn on the second day after intracerebral inoculation with 10^5 LD_{50} of virus. In spite of the high viral content of the brains before extraction, no agglutination was observed with the brain antigen under the conditions of the test. The antigen derived from serum, however, agglutinated chick cells at temperatures of 4°, 22° and 37°C, at an optimal pH range of 6.4 to 6.6 (Table 4).

The temperature and pH required for HA by Uruma antigen indicates that the virus falls into Casals' Group A. The characteristics of Uruma HA antigen closely resemble those of Mayaro,[3] Semliki Forest,[5] and Sindbis[2] antigens, in that variation in temperature had little influence on HA titer and the optimal pH for reactivity was near 6.4. Slightly higher HA titers were observed at 4° and 22°C than at 37°C; in this respect Uruma antigen differs from equine

TABLE 4

Hemagglutination by Uruma virus

Antigen source	Titer antigen obtained at indicated temperature and pH																		
	4°C							22°C							37°C						
	6.0	6.2	6.4	6.6	6.8	7.0	7.2	6.0	6.2	6.4	6.6	6.8	7.0	7.2	6.0	6.2	6.4	6.6	6.8	7.0	7.2
Suckling mouse brain (infectivity titer = $10^{-6.7}$/0.02 cc)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Suckling mouse serum (infectivity titer = $10^{-7.8}$/0.02 cc)	20*	20	160	80	40	0	0	0	10	40	160	10	0	0	0	10	40	80	0	0	0

* Reciprocal dilution of antigen producing complete agglutination of cells.

TABLE 5

Hemagglutination-inhibition tests with Uruma antigen and Groups A and B antisera

Antiserum	Serum HI titer* against	
	Homologous antigen	Uruma antigen
Group A		
Uruma	—	1280†
Mayaro	1280	320
Semliki	N. T.	80
Western equine encephalitis	640	20
Sindbis	N. T.	10
Eastern equine encephalitis	N. T.	10
Group B		
Japanese encephalitis	5120	<5
St. Louis encephalitis	2560	<5
Murray Valley encephalitis	5120	<5

* Sera tested against 4 units of respective antigens.

† Reciprocal of serum dilution.

encephalitis antigens which show greatest HA activity at 37°C.

Table 5 summarizes the results of hemagglutination-inhibition tests in which Uruma HA antigen was tested against hyperimmune mouse and guinea pig sera to 6 Group A viruses and 3 Group B viruses. It can be seen that the reactivity of Uruma antigen was inhibited to some extent by all of the Group A sera, but by none of the Group B sera. Maximal inhibition was produced by the homologous antiserum, with progressively less inhibitory activity displayed by Mayaro, Semliki Forest, Western equine encephalitis, Sindbis and Eastern equine encephalitis antisera. The HI tests thus confirmed the impression, formed from the neutralization test results, that Uruma virus is a member of Group A and is closely related to Mayaro and Semliki Forest viruses.

Complement-fixation studies. The HI test, because of its broad specificity, is undoubtedly the best method for demonstrating antigenic similarities among viruses of a given group. Conversely, the test usually fails to detect minor antigenic differences between viruses, and data derived therefrom should not be used as sole evidence for antigenic dissimilarity. Thus, although the HI data indicated considerable antigenic overlap among Uruma, Mayaro and Semliki Forest viruses, the differences observed were not interpreted as proof of true antigenic variation. Additional evidence for differences in the antigenic make up of these viruses was provided by cross complement-fixation tests.

Complement-fixation antigens, consisting of high speed supernates (RCF = 20,000 G for 1 hour) from 20 per cent infected suckling mouse brain suspensions in unbuffered saline, were prepared from Uruma, Mayaro, Semliki Forest, Sindbis and Western equine encephalitis viruses. The antigens were tested in checkerboard titrations against hyperimmune sera to each of the viruses. Antisera were derived from mice and guinea pigs immunized with 4 to 5 doses of complement-fixation antigen. Sera which possessed antibody to normal mouse brain in sufficiently high titer to interfere with the interpretation of results were discarded. Only sera with at least a 16-fold difference between nonspecific brain and specific viral antibody titers were used. Normal antigen and normal serum controls were incorporated into all tests, but for the sake of brevity have been excluded from the tables.

Tables 6 and 7 summarize the results of cross complement-fixation tests with 5 Group A viruses and antisera. Table 6 gives the titers of antigens and antisera when tested against 4 units of homologous and heterologous antigens and sera. Although comparisons of actual test titers reveal differences between all of the viruses, the differ-

TABLE 6

Antigenic relationships among 5 Group A viruses as revealed by cross complement-fixation tests

Antisera	Antigens*				
	Uruma	Mayaro	Semliki	Sindbis	WEE†
Uruma	128/32	32/16	32/32	16/8	16/8
Mayaro	8/32	16/16	4/32	4/4	4/8
Semliki	8/<4	8/<4	64/32	4/<4	16/8
Sindbis	4/4	4/<4	<4/<4	64/16	4/<4
WEE	<4/<4	4/<4	<4/<4	<4/<4	64/16

* Numerator denotes reciprocal antibody titer in presence of 4 units of antigen; denominator denotes reciprocal antigen titer in presence of 4 units of antibody.

† Western equine encephalitis.

TABLE 7

Antibody and antigen required for complete complement fixation (CF)

Antisera	Units of antibody required for complete CF in presence of 4 units of the following antigens:				
	Uruma	Mayaro	Semliki	Sindbis	WEE
Uruma	*1*	4	4	8	8
Mayaro	2	*1*	4	4	4
Semliki	8	8	*1*	16	4
Sindbis	16	16	NF (16)	*1*	16
WEE	NF (16)*	16	NF (16)	NF (16)	*1*
Antigens	**Units of antigen required for complete CF in presence of 4 units of the following antisera:**				
	Uruma	Mayaro	Semliki	Sindbis	WEE
Uruma	*1*	1	NF (8)*	8	NF (8)
Mayaro	1	*1*	NF (4)	NF (4)	NF (4)
Semliki	1	1	*1*	NF (8)	NF (8)
Sindbis	2	4	NF (4)	*1*	NF (4)
WEE	2	2	2	NF (4)	*1*

* No fixation with indicated units of antibody or antigen.

ences become more obvious when the data are presented in a somewhat different manner. Table 7 contains the same data, in which titers are assigned numbers corresponding to the units of antigen and antibody required for fixation. For example, the amounts of Uruma antibody required to fix complement in the presence of 4 units of Uruma, Mayaro and Sindbis antigens were 1, 4 and 8 units, respectively. Similarly, whereas 1 unit of Semliki Forest antibody was sufficient to give fixation with 4 units of homologous antigen, 8 and 16 units were required for fixation with 4 units of Uruma and Sindbis antigens. Using more potent Mayaro reagents furnished by Dr. Jordi Casals of the Rockefeller Institute (homologous antigen and antisera, titers of 1:32 and 1:128, respectively) essentially similar results were obtained. It is of interest to note that greater antigenic diversity was revealed by comparing the activity of each antiserum with constant amounts of homologous and heterologous antigens than by comparing antigen reactivity with constant amounts of antisera. Thus, while Uruma, Mayaro and Semliki Forest viruses appeared quite distinct by the former comparison, they seemed almost identical by the latter. This finding emphasizes the necessity of utilizing several serologic methods for determining similarities and differences in the antigen make-up of viruses within a given immunologic group.

Data derived from hemagglutination, hemagglutination-inhibition, complement-fixation and neutralization studies thus indicate that Uruma virus is a member of Casals' Group A, closely related, but not identical to, Mayaro virus and more distantly related to Semliki Forest virus and, to a lesser extent, to Sindbis virus. The neutralization test results in particular indicate that Uruma and Mayaro viruses are two distinct strains of this subgroup of Group A viruses.

ANTIBODY STUDIES ON HUMAN SERA FROM BOLIVIA

Virus-dilution neutralization tests were performed on: (a) acute and convalescent sera of the patient (Y.S.) from whom Uruma virus was isolated, (b) convalescent sera of 38 other Okinawan patients from whom acute phase sera were not obtained, and (c) sera from 21 healthy residents of Puerto Cespedes, Bolivia, who had not been involved in the jungle fever outbreak which afflicted Uruma colony. The results of these tests are summarized in Table 8.

Uruma virus was shown to be the cause of illness in the patient from whom it was isolated by the demonstration of a marked increase in serum-neutralizing antibody to the virus during convalescence. Sera collected from this patient on the first day, seventh week and fourth month after onset of symptoms, neutralized less than 8, more than 250,000 and 8,000 LD_{50} of Uruma virus,

TABLE 8

Neutralizing antibody to Uruma virus in sera from Bolivian residents

Sera	Logs of virus neutralized
Okinawan colonists (Uruma colony)	
Case Y.S.	
Acute (1st day of disease)	<0.9
Convalescent (7 weeks)	>5.4
Convalescent (4 months)	3.9
Case	
H.E., convalescent	>2.4
T.K., convalescent	>2.4
M.M., convalescent	>2.3
S.Y., convalescent	1.1
M.O., convalescent	1.3
33 cases, convalescent	<1.0
Healthy Bolivian residents (Puerto Cespedes)	
8 positive or equivocal	1.1->2.4
13 negative	<1.0

respectively. The virus was thus established as the cause of at least one case of jungle fever in the Okinawan colony. Convalescent sera from 3 of 38 other Okinawans, who experienced febrile illnesses similar to that of patient Y.S., contained significant levels of neutralizing antibody to Uruma virus. However, as no acute phase sera were obtained from these patients, a definitive etiologic diagnosis could not be established.

Preliminary information concerning the prevalence of infection with Uruma virus in this locality was derived from neutralization tests on a limited number of sera from healthy indigenous Bolivians. Sera from 8 of 21 (38 per cent) Indians and Caucasians living in Puerto Cespedes, a settlement 12 kilometers north of Uruma colony, possessed detectable antibody to the virus. These findings indicate that infection with Uruma virus, or a close antigenic relative, is fairly common among residents of the rain forest adjacent to Santa Cruz, Bolivia.

DISCUSSION

During the past decade, as a result of increased interest in the ecology and epidemiology of arthropod-borne viruses and the consequent establishment of new field laboratories throughout the world, the frequency of virus isolations from human, animal and arthropod sources reached an unprecedented high. Many "new" viruses have been described and, with the addition of new names to the list of recognized viruses, the identification of unknown isolates has become proportionately more difficult. In addition to the greater number of viruses with which each isolate must be compared, several other factors make the question of the uniqueness of a given isolate difficult to answer. Thus, the lack of a standard universally employed identification scheme, limited exchange of information among laboratories simultaneously engaged in identification work, and delays in publication of results are all conditions which demand that a virus may be designated as "new" only with certain reservations. Accordingly, such cautious terms as "presumably new" and "tentatively identified" have appeared in recent literature on virus characterizations. It is with such reservations, therefore, that we regard Uruma virus as a newly recognized and distinct viral entity.

The question of whether to assign the status of a new virus to an isolate closely related to another member of the same antigenic group was recently discussed in relation to Mayaro virus.[3] On the basis of neutralization test results, the Mayaro virus might have been regarded as identical to Semliki Forest virus. However, because definite antigenic differences between the two viruses were demonstrable by means of complement-fixation and hemagglutination-inhibition tests, the agents were considered distinct entities.

The classification of Uruma virus poses a similar problem. All of the serologic procedures employed in its identification indicated an antigenic relationship to Mayaro, Semliki Forest and Sindbis viruses. However, none of the tests revealed a complete antigenic overlap between Uruma virus and any of the other Group A agents studied. Because of the differences observed, particularly in the cross complement-fixation tests, Uruma virus has been regarded as a distinct agent. The position which it will eventually assume in a final definitive classification will be determined by further comparisons of the physical and chemical properties, host ranges and antigenic compositions of the Group A viruses.

SUMMARY

A newly recognized agent, named Uruma virus, was isolated from the blood of 2 febrile Okinawan colonists living in the rain forest of

eastern Bolivia in March 1955. Studies of its physical properties, host range and immunological characteristics have been described.

The virus was consistently pathogenic to infant mice, embryonated eggs and several tissue cell lines, but only weakly pathogenic for weanling mice, adult guinea pigs, hamsters and rabbits. Its particle size was estimated, by filtration and ultracentrifugation, to be about 25 mμ. It was found to belong to Casals' Group A, to bear a close antigenic relationship to Mayaro virus and to be more distantly related to Semliki Forest and Sindbis viruses.

Antibody studies indicated that: (a) Uruma virus was the cause of illness in the patient from whom it was isolated, and (b) infection with the virus, or a close antigenic relative, is fairly common among indigenous residents of the rain forest adjacent to Santa Cruz, Bolivia.

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ISOLATION OF A TYPE 2 DENGUE VIRUS BY USE OF HAMSTER KIDNEY CELL CULTURES*

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Hamster kidney cell (HKC) cultures as an additional tool for laboratory studies of certain of the arthropod-borne viral encephalitides have proved useful for the propagation of mouse-adapted laboratory strains of Eastern equine, Western equine, Venezuelan equine, St. Louis, Japanese B, Murray Valley and Ilheus viruses.[6] In addition, West Nile, Uganda S, Bunyamwera, Semliki Forest and dengue fever mouse-adapted viruses have been shown to produce cytopathogenic changes in HKC monolayer tube cultures.[1] More recently, Japanese B encephalitis virus has been isolated successfully in HKC from field specimens, and the sensitivity of the cultures demonstrated to be equal to or greater than that of suckling mice.[2] However, the changes elicited by the strains of dengue and St. Louis viruses tested were more subtle and difficult to detect. Hotta and Evans[4, 5] have reported that both type 1 and type 2 dengue viruses can be serially propagated in monkey kidney cell cultures but the cytopathogenic effects induced and the titers obtained leave much to be desired. During the course of studies designed to indicate the usefulness of the HKC in vitro method for routine application in public health laboratories, it was thought feasible to use the HKC cultures as an enrichment culture procedure to enhance the chance of recovering dengue virus from human blood in the more usual suckling mouse host recommended by Sabin.[8] It is the purpose of this report to record the details of the successful recovery by use of HKC cultures of a type 2 dengue virus from an acute phase human serum specimen which had been stored at CO_2 icebox temperatures for a period of 12 years.

* A portion of a dissertation submitted to the Graduate School of Public Health, University of Pittsburgh, in partial satisfaction of the requirements for the degree of Doctor of Science in Hygiene. This work was done under sponsorship of the Commission on Viral Infections of the Armed Forces Epidemiological Board, and supported in part by the Office of the Surgeon General, Department of the Army.

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MATERIALS

The preparation and maintenance of HKC monolayer tube cultures for routine laboratory use has been described in detail in a previous report.[1] The methods used were essentially identical to those currently in use for the routine processing of monkey kidney as recently summarized by Melnick.[7]

In attempts to isolate dengue virus, acute phase patient sera were kindly made available by Dr. W. McD. Hammon in whose laboratory this work was done. The histories of the 5 sera tested were somewhat similar but since only one strain was definitely isolated, this alone is presented. Dates and more details are presented in Figure 1. Serum from patient Brown-Neaves was sent from New Guinea to Dr. Hammon by Colonel C. G. Phillip. This was given 2 passages in human volunteers by Dr. Hammon, producing typical clinical dengue.[3] Acute phase serum from the second volunteer (Downs) was collected and frozen in a CO_2 icebox in 1945. This and other similar sera including the first human volunteer (Sotter) of this same Brown-Neaves line were used in these experiments. In previous attempts in this laboratory using these same sera after much shorter periods of freezing, no signs of illness had been manifest in inoculated mice. It was reasonable to assume that, even if marked cytopathogenic changes could not be used as an indication of viral increment, the HKC cultures might act as an enrichment medium and facilitate the subsequent recovery of virus in the suckling mouse.

EXPERIMENTAL PROCEDURES

The sera employed were identified as Downs, Van Deever, Sotter, Cowan and Keehan. These 5 sera were thawed at 37°C and two 10-fold dilutions were prepared in 10 per cent normal-calf-serum maintenance-medium diluent. Each of 3 HKC tube cultures was inoculated with 0.1 ml of the undiluted and with the 10^{-2} dilution of serum. The cultures were observed daily for 10

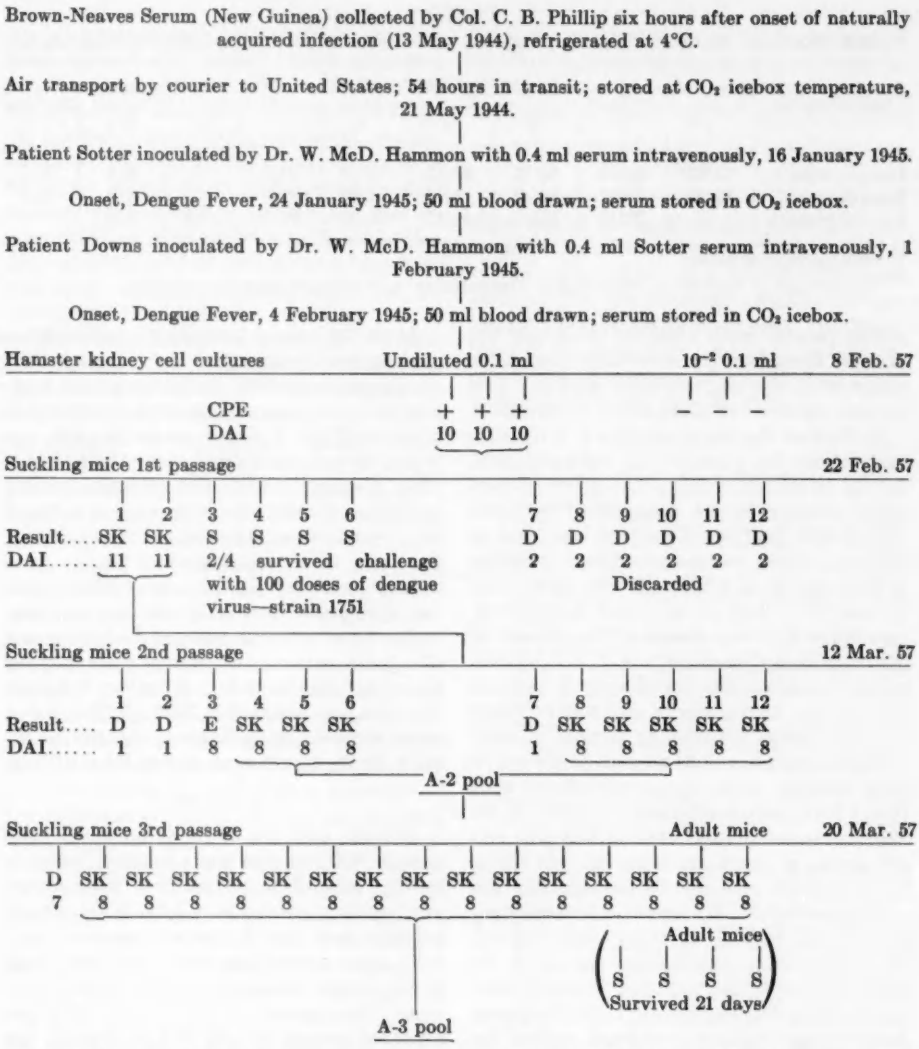
Brown-Neaves Serum (New Guinea) collected by Col. C. B. Phillip six hours after onset of naturally acquired infection (13 May 1944), refrigerated at 4°C.
Air transport by courier to United States; 54 hours in transit; stored at CO_2 icebox temperature, 21 May 1944.
Patient Sotter inoculated by Dr. W. McD. Hammon with 0.4 ml serum intravenously, 16 January 1945.
Onset, Dengue Fever, 24 January 1945; 50 ml blood drawn; serum stored in CO_2 icebox.
Patient Downs inoculated by Dr. W. McD. Hammon with 0.4 ml Sotter serum intravenously, 1 February 1945.
Onset, Dengue Fever, 4 February 1945; 50 ml blood drawn; serum stored in CO_2 icebox.
Hamster kidney cell cultures
Undiluted 0.1 ml
10^{-2} 0.1 ml
8 Feb. 57
CPE + + + − − −
DAI 10 10 10
Suckling mice 1st passage
22 Feb. 57
1 2 3 4 5 6 7 8 9 10 11 12
Result....SK SK S S S S D D D D D D
DAI..... 11 11 2/4 survived challenge with 100 doses of dengue virus—strain 1751 2 2 2 2 2 2
Discarded
Suckling mice 2nd passage
12 Mar. 57
1 2 3 4 5 6 7 8 9 10 11 12
Result.... D D E SK SK SK D SK SK SK SK SK
DAI..... 1 1 8 8 8 8 1 8 8 8 8 8
A-2 pool
Suckling mice 3rd passage
Adult mice
20 Mar. 57
D SK SK SK SK SK SK SK SK SK SK SK SK SK SK SK SK
7 8 8 8 8 8 8 8 8 8 8 8 8 8 8 8
Adult mice
S S S S
Survived 21 days
A-3 pool

FIG. 1. Flow sheet depicting recovery of Downs virus by use of hamster kidney cell cultures. CPE, cytopathogenic effect; DAI, day after inoculation; E, dead and eaten by mother; SK, sick—killed; S, survived; D, dead.

days for microscopic evidence of cellular degeneration.

On the 10th day following inoculation, 4 of the 5 sets of tubes (Downs, Sotter, Cowan and Keehan) which had received the undiluted serum inocula showed microscopic evidence of degeneration greater than that observed in the 6 control tubes. The fluids from all 5 inoculations with undiluted serum were pooled into individual lots by virus strain. This material was then ampuled, shell frozen and stored at CO_2 icebox temperatures. Four days later, aliquots of these pools were further diluted 10^{-1} and 10^{-3} and this first passage tissue culture material was then passed to a second set of HKC cultures, 3 tubes receiving 0.1 ml of each of the 2 dilutions. These

TABLE 1

Identification neutralization test Downs—7th passage virus versus dengue immune and normal monkey sera

Inactivated serum	Dilution tested (negative log)							MLD_{50} (negative log)	N.I.* logs
	2	3	4	5	6	7	8		
Dengue type 1..	12/12†	12/12	12/12	10/12	6/12	0/12		5.8	0.7
Dengue type 2..	12/12	12/12	10/12	2/12	1/12	0/12		4.5	2.0
Normal monkey.		12/12	12/12	12/12	10/12	2/12	0/12	6.5	

* Neutralization index.

† Numerator = number of mice dying; denominator = number of mice inoculated.

second passage tubes remained essentially unchanged throughout the subsequent observation period of 10 days and no further work was done in tissue cultures with these attempted isolations.

At the time the second passages in HKC were initiated, the first passage tissue culture material derived from tubes receiving the undiluted Downs serum as inoculum (i.e. a portion of the tissue culture fluid pool which had not been used as described above) was inoculated into 2 families of 2-day-old suckling mice. All mice of one family were found dead on the second day following inoculation and were discarded. The 6 mice of the other family remained well for 10 days following inoculation but showed signs of illness on the 11th day. At this time, 2 mice were sacrificed and their brains removed for passage to additional suckling mice. A 20 per cent suspension of these suckling mouse brains was diluted with 10 per cent normal-rabbit-serum nutrient-broth diluent containing 1,000 units of penicillin and 400 gamma of streptomycin per ml, was centrifuged at 2,000 rpm for 10 minutes, and the supernatant used to inoculate 2 additional families of baby mice. From this passage onward, the Downs virus was readily maintained by serial transfer in the brains of suckling mice. Figure 1 is a flow sheet recording the pertinent details of the isolation procedures utilized for recovery of the virus.

At the 4th passage level, 4 adult mice, in addition to the 2 families of suckling mice, were inoculated with 0.03 ml intracerebrally. The suckling mice sickened and began to die on the 7th day following inoculation, while the adult animals survived without evidence of illness for 21 days following inoculation. As serial passage continued, the incubation period shortened until the 7th passage virus induced illness and death in suckling mice in 5 to 7 days.

At the 7th passage level, the first attempt was made to identify this agent. Unfortunately, there were many nonspecific deaths among the suckling mice and accurate end-points could not be obtained. Type 2 dengue serum, however, appeared to have neutralized at least 100 LD_{50} of virus. A second identification neutralization test established the identity of this agent as a dengue fever type 2 virus. The details of this neutralization test, which was performed in the usual manner by mixing 0.15 ml each of diluted virus and appropriate inactivated monkey sera, incubating the serum-virus mixtures for 2 hours in a 37°C water bath, and inoculating these mixtures intracerebrally into each of 2 families of 6 suckling mice, are recorded in Table 1. The type 1 serum showed a neutralization index (NI) of 0.7 log, while the type 2 serum had an NI of 2.0 logs.

DISCUSSION

Although only one of the 5 attempted isolations in HKC cultures was adequately tested in suckling mice, it is evident from these experiments that by means of inoculating HKC cultures a dengue fever type 2 virus was recovered from acute phase human sera which had been stored at CO_2 icebox temperatures for a period of 12 years. The neutralization procedure with inactivated monkey serum used to identify the virus was intentionally chosen since it measures only the reaction of the virus with the heat stable antibody component and thus yields results which are free of the influence of the heat labile accessory factor which is necessary for the demonstration of maximum neutralization indices.[8] Concomitant isolation attempts directly in suckling mice were not carried out, but previous attempts in Dr. Hammon's laboratory to isolate and adapt this agent in mice had been unsuccessful. In view of the known lability of this virus at

incubator temperatures, it is unlikely that the virus survived the original 10-day incubation period in tissue culture without active infection and replication in the HKC culture. At the time the original isolations were attempted, the importance of adding stabilizing normal serum to the tissue culture fluids before harvesting and freezing had not been recognized[1] and the absence of its use lends credence to the belief that this represents the first recovery of dengue fever virus by the direct inoculation of a tissue culture system with infected human serum.

The recovery of the Downs strain of dengue virus by the use of HKC cultures represents an application of in vitro methods preceding animal inoculation. Such was recommended by Sanders and Alexander[9] for recovery of epidemic keratoconjunctivitis viruses in Maitland type embryonic mouse brain tissue cultures, and it is believed such methods deserve more extensive use in virology laboratories. Many of the arthropod-borne viruses have the capacity to infect and multiply in a variety of cell lines, even though cytopathogenic effects are not consistently induced in the monolayers. This capacity affords an opportunity for increasing the probability of recovery of virus from field-collected materials by an enrichment procedure analogous to techniques devised by the microbiologists for recovery of microorganisms from feces, soil or other materials containing only minute quantities of viable organisms. With appropriate modifications, such as repeated application of virus-containing materials to a monolayer of cells and the lapse of a suitable period of time to allow for absorption, much larger quantities of fluids could be tested for the presence of virus. Followed by a suitable period of incubation, these inoculated cell cultures should provide an enriched fluid which could then serve as starting material for the detection of viruses by the conventional means of inoculating susceptible animals.

It should be noted, however, that the effectiveness of such procedures would be limited by the toxicity and sterility of the materials to be tested. Therefore, the judicious use of centrifugation, filtration and dilution methods is indicated to overcome these objections and thus provide a more sensitive system for the recovery of virus.

SUMMARY

Attempts to recover dengue virus from 5 acute phase human serum specimens which had been stored at CO_2 icebox temperatures for a period of 12 years were carried out in hamster kidney cell cultures. The one successful isolation attempt yielded a type 2 dengue fever virus. The importance of the procedures used for increasing the probability of recovery of viruses from virus-containing materials was discussed.

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ADENOVIRUSES ISOLATED FROM SAUDI ARABIA

I. Epidemiologic Features*

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In the course of an investigation of the etiology of trachoma and certain other diseases of the eye in the Eastern Province of Saudi Arabia, a large number of viral agents have been isolated. During the period June 1955 to September 1957, approximately 5000 individuals were examined for eye pathology, over 90 per cent of whom had clinical evidence of trachoma. Approximately 1000 conjunctival scrapings were obtained for virus isolation attempts of which 948 were tested in tissue culture. By September 1957, 67 strains of viruses were isolated, of which 2 proved to be Coxsackie strains B1 and B2 while all the remainder fell into the group known as adenoviruses. An abstract of the findings from the summer of 1955, when the first 12 virus strains were isolated has been published.[9] It is the purpose of this paper to present the epidemiologic features relating to the adenoviruses which were obtained between June 1955 and September 1957 in the Eastern Province of Saudi Arabia.

The environment and population. The areas covered in this study are in the general vicinity of Dhahran in the Eastern Province of Saudi Arabia. Here there are two main oases, Qatif to the north on the Persian Gulf, and Al Hasa, inland to the south and west. In each of these oases there are numerous small garden villages as well as larger towns and cities. The remainder of the province is desert. The climate is extremely hot during the summer, from April to September, the average seasonal maximal temperature being 100°F (38°C). It is relatively cool during the winter when average monthly minima fall to the vicinity of 60°F (15°C). Rainfall averages less than three inches per year and occurs during the winter.

The people who were included in this study were Saudi Arabs who either lived in permanent settlements in and about the oases or were nomadic Bedous who encamped temporarily near the oases during the arid summer months and roamed the deserts during the winter grazing season. On the coast fishing is an important income-producing occupation. Many men in both groups are present or past employees of the Arabian American Oil Company (Aramco). Sanitation and living conditons generally are primitive throughout the Eastern Province. Two exceptions, in addition to the Aramco community, are the costal towns of Dammam and Al Khobar which have grown rapidly in recent years in relation to the industrial developments. Living conditions in these two communities are considerably above the average for Eastern Saudi Arabia. Medical care has been unavailable to the general population of the oases until very recently, although the Aramco Health Center has developed excellent facilities for its employees and their families. In its efforts to establish a program of preventive medicine and public health in the community, the Ministry of Health of Saudi Arabia has been assisted by the Aramco Medical Department. The geography, climatology and ethnology of the Eastern Province have been described in some detail by Tarizzo.[17] The area of the study is shown in Figure 1.

Several procedures were used to examine patients and collect specimens:

a. Patients who attended the outpatient clinics of the Aramco Medical Department Health Center were examined and specimens obtained from those who appeared to have eye pathology.

b. Surveys were made of school children or of the general population in towns and villages; in such cases specimens were taken at random from all children who presented themselves.

c. Special clinics were set up in some villages

* This research was supported in part by the Arabian American Oil Company.

FIG. 1. Study area in Eastern Saudi Arabia. 1, Agrabia; 2, Anech; 3, Awamiyah; 4, Hazim Oujam; 5, Hazim Um Es Sahich; 6, Khataishiya; 7, Qatif; 8, Safwa; 9, Saihat; 10, Subaika.

where attempts were made to follow various individuals. In this case serial specimens were often obtained from single patients.

School surveys and special clinics were conducted in two areas: on Tarut, an island in the Persian Gulf near Qatif consisting of a garden area and several small villages, and in the village of Um es Sahich, an inland garden area just north of the Qatif oasis. General population surveys were made in several villages including Anek, Hazim um es Sahich and Hazim Oujam (all temporary Bedou encampments) and Khataishiya, a permanent town, all in the Qatif area. In the vicinity of the Al Hasa Oasis, Mutairifi and Al Umran are remote, difficultly accessible villages on the outskirts of this oasis.

It was discovered very early in the course of this study that most of the population over the age of 6 years suffered from chronic advanced trachoma with pannus and scarring. It was therefore decided to concentrate the search for etiologic agents as much as possible on children rather than adults. In very general terms it could be said that by the MacCallan's classification most children up to 6 years had trachoma II; those from 6 years to early adult life had trachoma III; and that most adults had trachoma III or trachoma IV. It was also repeatedly noticed that the disease appeared to be milder in Bedous than in the settled populations. Detailed clinical notations were available on 48 of the 65 adenovirus isolations. Twenty-four of these patients were found to have trachoma in stage I or stage II with follicles; 26 were observed to have obvious conjunctivitis and over half of these manifested marked edema and swelling of the conjunctivae and lids, usually with profuse exudate, photophobia and lachrymation.

MATERIALS AND METHODS

Eye scrapings. The standard technic for taking a conjunctival specimen consisted of everting the upper lid and scraping along the tarsus with a stainless steel or chrome plated instrument shaped like a golf stick. No anesthetic was used. A portion of the material was scraped off onto a small cover slip which was placed in a vial containing a small amount of preserving fluid. The specimen was transported chilled to the central laboratory where it was either inoculated directly into tissue culture or frozen at −70°C. Approximately 85 per cent of the specimens were frozen and tested at a later time. At each examination a small portion of the scraping was usually smeared on a glass slide and stained by the Giemsa method for detection of elementary bodies and inclusions.

Preserving fluids. Several types of fluids were used, the most common being sucrose PG[3] and a mixture of 20 per cent inactivated horse serum in MS.[16] Occasionally MS without serum was used and, more rarely, inactivated human serum was substituted for horse serum.

Tissue cultures. Continuously cultivated human cell lines of the liver, conjunctiva,[4] or LN[3] strains were used in all isolation studies. These cell lines were maintained in a medium consisting of 20 per cent inactivated horse serum in MS with penicillin and streptomycin in amounts varying between 2 and 100 units. Media were replaced every 3 to 4 days.

Virus isolations. Fresh or frozen conjunctival scrapings were introduced into a roller tube of cells by washing the medium in the vial containing the scrapings and then replacing it in the tissue culture tube. Such cultures were held for 50 days at which time they were considered negative if no cytopathogenic effects were ob-

served. Adenoviruses made their appearance as early as 7 days and as late as 45 days after inoculation.

Subsequent to much of this work it was shown by Chang and Geyer[5] that sera from the horse used in the isolation studies reported herein were inhibitory to one strain of adenovirus type 3. It is therefore possible that some viruses were missed if they had been present in the eye in low concentrations.

Virus identification. All virus strains were tested by complement fixation reaction, using the virus as antigen and known positive adenovirus antisera. The 2 viruses which were typed as Coxsackie B1 and B2 had an obviously different cytopathogenic pattern from all the other strains. Typing of the adenoviruses was performed by neutralization with specific rabbit antisera[14] and also by the use of hemagglutination and hemagglutination-inhibition.[12] The virus prototypes 1 through 12 were obtained early in 1956, from Dr. Robert Huebner. Prototypes 15, 16, 17, strain 587, and strain 931 were first isolated in Saudi Arabia in 1955.[9, 15] Prototypes 13, 14, 18 and strain BAR-2[12] were obtained from Dr. Leon Rosen. Strains 1645 and 2711 were isolated in Saudi Arabia in 1956.

RESULTS

Adenovirus by season. Table 1 shows the distribution of adenovirus isolations by month and year. During the summer months of April through September, 60 of the 65, or 92 per cent, of the viruses were isolated. These 60 isolates were made from a total of 451 attempts, an isolation rate of 13.3 per cent. In contrast, the winter rate from 495 attempts was only 1 per cent. The summer of 1957 saw a very low isolation rate (4.1 per cent) when compared to 1955 (12.8 per cent) and 1956 (17 per cent). It is believed that this can be explained by the fact that the majority of the specimens obtained in 1957 were from blanket surveys of remote villages, whereas in the other two

TABLE 1

*Isolation of adenoviruses by month, all ages, 1955–57**

	1955	1956	1957	Total	Isolation rate (%)
January		2/140	0/5	2/145	1.4
February		1/222		1/222	0.5
March			0/31	0/31	0.0
April			2/28	2/28	7.2
May		4/15		4/15	26.6
June		12/57	0/12	12/69	17.8
July	2/19	9/56	2/41	13/116	11.2
August	7/52	17/77	0/17	24/146	16.8
September	3/23	2/54		5/77	6.5
October	1/4	1/57		2/61	3.3
November		0/32		0/32	0.0
December		0/4		0/4	0.0
Total	13/98	48/714	4/134	65/946	6.9

* Numerator = number of adenovirus strains isolated; denominator = number of isolation attempts.

TABLE 2

Isolations of adenoviruses by age, 1955–57

Age	Summer				Winter			Total both seasons
	1955	1956	1957	Total	1955–56	1956–57	Total	
0–3 months	0/3	2/11	0/4	2/18	0/17	0/2	0/19	2/37
4–6 months	3/11	10/33	0/7	13/51	1/15	0/7	1/22	14/73
7–11 months	3/16	16/41	1/8	20/65	0/24	0/12	0/36	20/101
12–23 months	3/17	8/37	2/14	13/68	1/39	0/15	1/54	14/122
24–35 months	1/11	3/44	1/14	5/69	0/30	1/16	1/46	6/115
3–5 years	0/14	2/49	0/25	2/88	0/71	0/46	0/117	2/205
6–10 years	2/14	2/32	0/10	4/56	0/114	0/18	0/132	4/188
+10 years	0/5	0/4	0/2	0/11	0/53	0/10	0/63	0/74
Unknown	0/3	1/8	0/14	1/25	2/3	0/3	2/6	3/31
Total	12/94	44/259	4/98	60/451	4/366	1/129	5/495	65/946

years, even in survey work, active ophthalmic disease was sought out. This is shown by a comparison of clinic and survey data in Table 3. The fact that 52 per cent of isolation attempts were made during the winter months rules out the possibility that the preponderance of positive isolations was due to more intensive efforts during the summer.

Adenovirus by age. A second striking epidemiologic feature of this study is illustrated in Table 2. Of the 62 isolations where the age was known, 81 per cent were from children under the age of 2 years, and 90 per cent under the age of 3 years. This is not due to larger numbers of attempts in the very young age groups as is shown by the fact that the isolates were obtained from less than half of all attempted isolations. Another interesting feature of the age patterns is illustrated in

TABLE 3

Isolations of adenoviruses in 1955–57 in clinics and surveys

	Summer				Winter			Total both seasons
	1955	1956	1957	Total	1955–56	1956–57	Total	
Clinics		—						
Dhahran Health Center	12/94	18/56	2/27	32/177	3/193	0/21	3/214	35/391
Agrabia	1/1	1/5		3/6	0/3		0/3	2/9
Awamiya		1/1		1/1	0/1		0/1	1/2
Dammam	1/19	5/13	1/13	7/45	1/41	0/2	1/43	8/88
Hofuf	1/7			1/7	0/6	0/1	0/7	1/14
Khobar	1/4	0/4	0/2	1/10	0/11	0/1	0/12	1/22
Qatif	2/6		0/1	2/7	0/10	0/5	0/15	2/22
Saihat	0/1	1/4	1/2	2/7	0/22		0/22	2/29
Subaika	0/1	1/2		1/3	0/6		1/6	1/9
Thuqba	2/13	4/13	0/3	6/29	1/59	0/4	1/63	7/92
Miscellaneous villages:								
Al Hasa Oasis	0/2			0/2	0/13		0/13	0/15
Qatif Oasis	0/3			0/3	0/6		0/6	0/9
Tarut Oasis					0/5		0/5	0/5
Other*	4/37	5/14	0/6	9/57	1/10	0/8	1/18	10/75
Tarut Island		10/79	0/1	10/80		1/33	1/33	11/113
Um es Sahich		4/44		4/44		0/45	0/45	4/89
Totals, all clinics	12/94	32/179	2/28	46/301	3/193	1/99	4/292	50/593
Surveys								
Anek		4/25	0/11	4/36		0/5	0/5	4/41
Hazim Oujam		1/7		1/7		0/10	0/10	1/17
Hazim Um es Sahich		1/14		1/14				1/14
Khataishiya		5/21	0/2	5/23		0/2	0/2	5/25
Mutairifi		1/8		1/8		0/5	0/5	1/13
Tarut					1/68		1/68	1/68
Um es Sahich			0/14	0/14	0/105		0/105	0/119
Al Umran			2/33	2/33				2/33
Miscellaneous villages:								
Al Hasa Oasis			0/10	0/10		0/8	0/8	0/18
Other*		0/5		0/5				0/5
Totals, all surveys		12/80	2/70	14/150	1/173	0/30	1/203	15/353
Total	12/94	44/259	4/98	60/451	4/366	1/129	5/495	65/946

* Includes data from patients whose current residence was not specified on the original data sheet.

TABLE 4

Adenovirus isolations in Dammam and Thuqba, Saudi Arabia, 1955–1957

	No. of isolations		Total No. (isolations/ attempts)	Adenovirus types isolated	
	Summer	Winter		Summer	Winter
Dammam	7/45	1/43	8/88	15, 8, BAR-2, 4, 8, 17, 15	1645
Thuqba	6/29	1/63	7/92	17, 931, 15, BAR-2, 4, 15	7

TABLE 5

Adenovirus types isolated, 1955–1957

Type	By Age								
	0–3 mos.	4–6 mos.	7–11 mos.	12–23 mos	24–35 mos	3–5 yrs.	6–10 yrs.	Unknown	Total
3		2	1	1		1	3		8
4		2	1				1		4
6			1					2	3
7		2							2
8	1		6	3					10
15		1	2	2					5
16			1						1
17		3		4	1				8
587		1	1		2				4
931		1	3						4
1645				1					1
2711					3	1			4
BAR-2	1	3	2	3				1	10
Untyped				1					1
Total	2	15	18	15	6	2	4	3	65

Type	By locality									
	Anek	Dammam	Khatai-shiya	Qatif	Tarut	Thuqba	Um Es Sahich	Al Umran	Other	Total
3					5		1		2	8
4	1	1				1			1	4
6					1				2	3
7				1		1				2
8		2	2	1	2				3	10
15		2				2		1		5
16									1	1
17		1			2	1	1		3	8
587								1	3	4
931					1	1			2	4
1645		1								1
2711	2						1		1	4
BAR-2	1	1	3		1	1			3	10
Untyped							1			1
Total	4	8	5	2	12	7	4	2	21	65

TABLE 6

Adenovirus isolations on Tarut Island, June 1956 to March 1957

	Darin	Adenovirus type	Rubaiyir	Adenovirus type	Tarut	Adenovirus type	Total*
June			1/4	3	2/4	3, 3	3/8
July			0/8		2/4	3, 8	2/20
August			2/5	8, 931	3/13	3, 17, BAR-2	5/25
September			0/6		0/13		0/22
October	1/6	17			0/6		1/12
November	0/2		0/2				0/4
December					0/3		0/4
January			0/4		0/1		0/5
March			0/2		0/4		0/8
Total	1/8		3/31		7/48		11/108*

* Included in the totals are 14 negative isolation attempts from Senabis and 7 from Zoor, as follows: Senabis, July 0/1, August 0/7, September 0/3, December 0/1, March 0/2; Zoor, July 0/7.

Table 5. Four of 8 type 3 isolations were obtained from children between the ages of 3 and 6 years, while 52 of the remaining 54 isolations were obtained from children under 3 years of age. The youngest infant from whom a virus was isolated was 68 days old, while the oldest child was 6 years.

The 3 isolated viruses for which there is no age information were obtained from pools of specimens, one from a pool of 20 clinic children from several villages in the Qatif Oasis, ages 4 to 8 years, another from 20 school children on Tarut Island, ages 7 to 14 years, and the third from 6 specimens from the Qatif Oasis area, ages 5 months to 3 years.

Adenovirus by locality and type of collection. The data in Tables 3 and 6 indicate that, with the possible exception of Khataishiya and Tarut, it is unlikely that the numbers of viruses isolated were due to epidemics in any one area. For example, of 8 isolations from Dammam there were 6 different virus types. The two strains of type 8 were obtained a month apart while the two strains of type 15 were obtained in different years. Similarly 6 of the 7 isolates from Thuqba were of different types and the two strains of type 15 were in different years (see Table 4). In surveys in Khataishiya, 3 type BAR-2 viruses were obtained within a period of one week on May 31 and June 7, 1956. Two months later, on August 9, 2 type 8 viruses were isolated. The small clinic set up on Tarut Island was visited at intervals of one to four weeks. Attempts were made to obtain serial specimens on individuals; of 73 individuals followed, specimens were obtained once from 50 children, twice from 24, three times from 30 and four times from one. There are five small towns on Tarut Island, Tarut itself in the center of the oasis, Darin, Senabis and Zoor on the treeless shore, and the tiny, inland village of Rubaiyir on the outskirts of the oasis. The data for the period when this clinic was active, June 1956 to March 1957, are given in Table 6. It seems possible that there was a small epidemic due to type 3 adenovirus in progress during June, July and August.

Adenovirus types. Table 5 shows the various types obtained and their frequency. Types 3, 8, 15, 17 and strains 955 and BAR-2 accounted for 41 of the 65 isolations or 61 per cent. It seems interesting that only two strains of type 7 and four of type 4 were obtained. Types 1, 2, 5, 10 to 14, and 18 were not encountered. In the neutralization tests for type 8 it was found that type 8 antisera would completely inhibit the cytopathogenic effect (CPE) produced by these strains. It was also noted that the CPE was considerably delayed by type 9 antisera, indicating some antigenic overlap. From 8 patients, scrapings were made of each eye and treated as separate entities. In each case the same virus type was obtained from each eye. Seven of these were isolated directly, and one from frozen, stored material. In the case of one type 4 isolation, the virus was reisolated from a second scraping taken after an interval of three days. This was the only case where a virus isolation was made a second time after the initial isolation;

in no case, therefore, despite serial attempts on a number of children, was it possible to obtain more than one adenovirus type from a single individual.

Two previously undescribed strains were encountered in 1956; strain 1645 which appears to be the same as one received from the U.S.S.R by Dr. Leon Rosen[13] and four representatives of strain 2711. One adenovirus remains completely untyped and uncharacterized.

Serology. From the outset it was recognized that paired serum samples on individuals from whom isolations were made would be highly desirable. Unfortunately, despite strenuous efforts it proved impossible to obtain blood samples from children in Saudi Arabia under the conditions of our study. The only data on antibodies to adenoviruses among Saudi Arabs were obtained from a group of patients admitted to the hospital in Dhahran, for treatment of various disorders other than trachoma. Single serum specimens from 78 Saudi Arab adults were available: 23 were negative at 1/5 in the complement fixation test; 55 had titers of 1/5 or higher, when tested against types 3 or 4 antigens.

DISCUSSION

Although the present study was concerned with trachoma and related diseases of the eye rather than respiratory disease, it is interesting to compare the findings reported herein with other accounts of adenovirus isolation. Adenovirus infection in the United States has generally been associated with epidemics of respiratory disease and pharyngo-conjunctival fever in both military and civilian populations.[1, 7] These have been described as most commonly due to types 3, 4, and 7 and have occurred at all seasons of the year in both adults and children. A recent report[8], presents a somewhat different picture of the epidemiology of adenovirus infections when virus isolations from throat swab specimens were attempted weekly in a population of children in an orphanage: the predominant types were 1, 2 and 3; more rarely 5, 10 and 13, which appeared to cause small epidemics at all seasons of the year. The marked seasonal incidence in Saudi Arabia and the predominance of isolations from children in the youngest age groups would indicate a different epidemiologic milieu in Saudi Arabia, although the age pattern bears similarities to the orphanage study cited by Huebner *et al.*[8] Furthermore, types 1, 2 and 5 which are common in the United States were never isolated in Arabia, while types 15, 16, 17 and several new types which were common in Arabia have not yet been reported in the United States. Apparently types 3 and 8, and strain BAR-2 are frequently found in both areas. It seems probable that children in Saudi Arabia are coming in contact with a spectrum of adenoviruses in infancy. Immunity to this group of viruses may be widespread by the age of 3 years, thus reducing the likelihood of large epidemics due to these agents among the adult population.

The possible importance of differences in housing and in living conditions was considered by contrasting Dammam and Thuqba which are treeless communities near Dhahran, the former representing a comparatively modern Saudi Arab city of 25,000 people with above average living standards and the latter a community of about the same size where conditions are in sharp contrast to Dammam. The adenovirus isolation experience was virtually the same in these two communities.

The individuals from whom virus isolations were attempted were also studied to determine the bacterial flora of the eye[11] and the occurrence of the typical inclusions associated with classic trachoma.[10] There was no correlation between either of these and the isolation of adenoviruses as reported herein. It has been maintained by several investigators that secondary bacterial infection is an important feature complicating the disease picture of trachoma. The findings reported in this paper suggest that the role of adenoviruses deserves careful evaluation in view of their rather common occurrence in the eyes of Saudi Arabs. Other observations[6] raise this issue even more sharply, and serve to justify continued search to identify the part played by adenoviruses in the pathogenesis of the clinical complex of eye disease which results in impaired vision. Whether the adenoviruses and certain bacterial species play an important role in enhancing the virulence or increasing the pathogenicity of a specific trachoma virus remains to be elucidated.

At the beginning of this study, it was considered highly desirable to obtain acute and convalescent sera on patients from whom viruses were isolated. To do this would have necessitated taking blood samples from all those from whom eye scrapings were sought; it would then have

been necessary to obtain follow-up convalescent samples, virtually all of which would have been from small children. Despite numerous attempts to obtain such specimens, this feature of the study was reluctantly abandoned in the interest of maintaining friendly relations and rapport with the villagers.

In retrospect the general picture of the epidemiology of adenoviruses in Saudi Arabia would have been more complete if other types of specimens such as nasopharyngeal washings and stool samples had been obtained. It is to be emphasized that all viruses which were isolated in this study were derived from the conjunctivae.

The frequent occurrence of type 8 prompted careful scrutiny of the clinical records for data on possible correlation with corneal opacities. The latter were not observed at the time the specimens were taken in any of the subjects from whom type 8 strains were isolated.

SUMMARY

Three years' experience in attempting to isolate viruses from conjunctival specimens from the autochthonous population of Eastern Saudi Arabia is described. From several thousand examinations, material for virus isolation was obtained from approximately 1000 individuals. Nine hundred forty-eight (948) specimens were tested in tissue culture and yielded two Coxsackie viruses, B1 and B2 and 65 adenovirus strains which fell into at least 13 different types. Of the 64 typed strains, the most common types were types 3, 8, 17 and strain BAR-2. Type 4 was isolated only three times and type 7 twice.

During the course of this study, several types of adenoviruses were isolated in Saudi Arabia which had not been encountered elsewhere. The new viruses are types 15, 16 and 17,[9] and the strains designated 587, 931, 1645 and 2711, which have not been assigned type numbers as yet.

There was a marked seasonal incidence in the isolations of adenoviruses; 92 per cent of the 65 strains being obtained during the summer months of April through September. Only five strains were isolated from October through March, although more than half the specimens were obtained during this period. Despite the fact that over half the attempted isolations were in the age group 3 years and over, 81 per cent of the isolations came from children under 2 and 90 per cent from those under 3 years. No clear cut widespread epidemics due to types 3, 4 and 7, were encountered in this study of the agents recoverable from the conjunctivae of Saudi Arabs. Brief consideration is given to the possible role of adenovirus infection in the clinical complex which is diagnosed as trachoma in Eastern Saudi Arabia.

ACKNOWLEDGMENTS

This study was the product of the combined efforts of a large number of individuals in Boston and in Saudi Arabia. The authors wish particularly to acknowledge the contributions of Miss Jane Driscoll and Miss Charlotte Smith for their technical assistance, Drs. M. L. Tarizzo, A. T. Hanna and R. L. Nichols for the collection of many of the field specimens, Drs. V. Young, E. Schurink and A. Yousef for their clinical help and understanding cooperation. The authors are grateful to Drs. R. C. Page, R. H. Daggy and T. C. Alexander of the Aramco Medical Department for their assistance, advice and encouragement. In the typing and classification of some of the viruses, the facilities and experience of Drs. L. Rosen, W. P. Rowe and R. L. Huebner at the National Institutes of Health were invaluable.

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ADENOVIRUSES ISOLATED FROM SAUDI ARABIA

II. Pathogenicity of Certain Strains for Man*

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The preceding paper of this series[1] reported the epidemiological features associated with the isolation of adenoviruses from Saudi Arabia. The agents were obtained from the conjunctivae of patients who had clinical findings consistent with the diagnosis of trachoma. These observations raised the question as to the role of Saudi Arab strains of adenoviruses in the clinical picture of trachoma. This report describes the clinical, laboratory and histological findings of three blind children infected with three different strains of adenoviruses isolated from Saudi Arabia.[1] The findings are compared with those of two additional blind children infected with conjunctival scrapings taken directly from cases of clinical trachoma, stage II.

METHOD OF INVESTIGATION

Five children were selected for this study on the basis of total blindness and the absence of conjunctival pathology. Preinfection studies consisted of detailed ophthalmological examination, cytological examination of conjunctival smears, and bacteriological and virological cultures of the conjunctival secretions. Saudi Arab adenovirus strains used for the inoculation of the patients were bacteriologically sterile lines maintained by serial transfers in human conjunctival cells[3] propagated in the manner noted below. The infecting strains were Dh55-79, type 16; Dh55-22, type 17; and Dh55-92, designated "931" which did not correspond to any of the types as recognized by Rowe *et al.*[6] Infection was accomplished by dropping approximately 0.05 ml of the culture fluid into the conjunctival sac of each eye followed by gentle rubbing of the inoculum into the palpebral conjunctiva with a sterile cotton swab. It is estimated that approximately 100 tissue culture ID_{50} doses were contained in this volume of inoculum, prior to the lyophilization which was performed in order to transport the strains to Hong Kong.

Following infection, the patients were isolated for 20 to 30 days and were examined at intervals of 1 to 3 days until acute signs and symptoms subsided and then at monthly intervals for 3 to 6 months. Cytological, bacteriological and virological studies were made of the conjunctival scrapings during the period of isolation. Blood samples were taken at intervals of 2 to 8 weeks. From each case in the series, a small biopsy of the left upper fornix was taken on the second to the fourth day after onset of signs of inflammation. To avoid possible complicating factors introduced by repeated mechanical trauma, all conjunctival scrapings were taken from the left eye of each patient.

The bacteriological studies consisted of direct plating on nutrient agar containing 10 per cent rabbit blood. The inocula consisted of swabs of the left upper fornix. Most of the cultures gave rise to a few colonies of alpha hemolytic streptococci and/or nonhemolytic staphylococci. The other cultures were sterile.

The virological studies consisted of direct inoculation of conjunctival scrapings into cultures of human conjunctival cells[3] nourished in a nutrient medium consisting of 20 per cent inactivated horse serum in Eagle's basal medium.[5] Cultures were observed for at least 50 days to detect the appearance of characteristic cytopathogenic changes.

Neutralizing and complement-fixing antibodies against the infecting strains of adenovirus were measured according to the method of Rowe *et al.*[7] In order to produce characteristic cytopathogenic changes on the third to fifth day after inoculation, the amounts of virus employed in the neutralization tests were about 100 ID_{50} for type 16; 1000 ID_{50} for type 17; and 3000 ID_{50} for strain "931."

RESULTS

The findings were presented under three separate headings: clinical, laboratory and histological.

* This work was supported in part by the Aramco Trachoma Research Program, Arabian American Oil Company.

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Clinical Results

Case No. 1. Patient, aged 16, with bilateral staphyloma was infected with adenovirus type 16. Two days following infection, conjunctival injection and slight mucoid discharge were first noted. By the 4th day, signs of acute follicular conjunctivitis were present; these were intense injection of palpebral and bulbar conjunctivae, the development of papillary hypertrophy, and formation of follicles in both upper and lower conjunctivae, and the presence of a moderate amount of mucoid discharge. By the 6th day vascular injection was visibly less while the follicles remained unchanged. Signs of acute inflammation continued to subside and by the 12th day, there was little conjunctival injection. The follicles at this stage were small, discrete, regular in size and distributed equally over upper and lower conjunctivae. One month after infection numerous small discrete follicles were still present in both the upper and lower fornices; these findings were still observed at examinations made on the 2d, 3rd and 6th months. The patient remained afebrile while hospitalized during the first 4 weeks. No lymphadenopathy or pharyngeal injections were noted. Since both cornea were completely scarred, possibly due to previous severe keratomalacia, corneal examination was not informative.

Case No. 2. Patient, aged 17, with staphyloma of both eyes, was infected with adenovirus, strain "931." The development of signs and symptoms were similar to Case No. 1 except that they were more severe and persisted longer. When last seen 27 months after infection, the conjunctivae of both eyes were normal.

Case No. 3. Patient, aged 13, with adherent leucoma and applanatio corneae, was infected with adenovirus type 17. The signs and symptoms of follicular conjunctivitis were again similar to Case No. 1, except for greater severity and longer duration.

Case No. 4. Patient, aged 7, with amblyopic nystagmus and surgical aphakia, but normal conjunctivae and corneae, was infected on the left eye with inclusion positive conjunctival scrapings, which had been frozen and thawed once, from a local case of trachoma, stage II. Mild discharge and congestion were first noted on the 4th day. Hypertrophy and follicles were seen in tarsal conjunctiva and fornices on the 6th day. By the 8th day, conjunctival injection began to subside. The clinical manifestations continued to subside for the next 7 weeks. On about the 56th day, there was a "relapse" involving the right as well as the left eyes. Clinical signs consisted predominantly of injection, hypertrophy and a few follicles. Vascular injection subsided readily. Similarly "relapse" occurred on the 7th and 8th months. Cornea remained negative throughout. When last seen on the 26th month, there were hypertrophy and follicles but no scar in the upper lids.

Case No. 5. Patient, aged 8, with postmeningitic optic atrophy of both eyes, was infected with inclusion negative conjunctival scrapings from a local case of trachoma, stage II. Three days later mild mucopurulent discharge, papillary hypertrophy, isolated follicles in tarsal conjunctivae and clusters of follicles in the fornices were noted. The upper conjunctivae were apparently more involved than the lower. The follicles varied in size and were surrounded by edema and vascularizations. Signs of infection were most severe on the 6th day, after which they gradually subsided. Transient haziness of both upper limbi, visible only through slit-lamp examination, were first seen on the 12th and 14th days for the left and right eyes, respectively. The corneal lesions were subepithelial but without new vascularization. They extended slightly beyond the loop palisade. When seen on the 30th day, the corneae were entirely negative. On the last visit, 25 months after infection, there were a few follicles in the tarsal conjunctiva and fornices and scattered area of papillary hypertrophy in the upper lids. There was no scar. The corneae were negative.

Laboratory Results

Pertinent laboratory data of cases 1, 2 and 3 are summarized in Table 1. Neutralizing antibodies against infective strains were demonstrable in all 3 patients following infection. Of interest are the results of virus isolation: virus was readily reisolated during the acute phase for Case No. 1, virus was isolated on the 10th and 12th days after onset of inflammatory signs but not earlier for Case No. 2, and no virus was isolated throughout the whole course for Case No. 3. Conjunctival smears revealed the presence of a few polymorphonuclear leucocytes.

Laboratory findings for Cases No. 4 and No. 5 were completely negative. At no time could inclusions or elementary bodies be demonstrated (these smears had been examined in the field as

TABLE 1

Summary of pertinent laboratory data of Cases 1, 2 and 3

Day after infection	Conjunctival smear	Isolation of adenovirus	Antibodies vs. infecting virus	
			neutralizing	C.F.
Case 1				
pre	−*	−	<1	<10
4	−	+		
6	−	+	<1	<10
20	−	−	<1	
25	−	−	16	20
40				40
Case 2				
pre	−	−	<1	<10
3	−	−		
6	−	−		
10	−	−		
12	−	+		<10
14	−	+		
32		−	1	20
60			1	
Case 3				
pre	−	−	<4	80
3	−	−	<4	80
6	−	−		
9	−	−	4	40
11	−	−		
14	−	−		
28	−	−	64	160

* No inclusion or elementary bodies could be found; the presence of some polymorphonuclear leucocytes was disregarded.

well as in the Harvard Laboratory by three different investigators). No cytopathogenic agents were isolated from the infecting materials, either from the donor patients (from whom the infecting materials were taken) or from the patients who received the material, at various intervals after onset of inflammatory signs.

Histological Findings

The biopsies from the five cases were fixed in formalin and prepared for microscopic examination by the standard procedures for pathological specimens. The sections were studied by three pathologists. The consensus was that there were no significant differences in histopathology to be noted among the five biopsies. The common finding on all of the specimens was that of an irregular epithelium and stroma. Scattered lymphocytes were present in both levels. In all of the specimens epithelial cells appeared to have been dispersed downward into the stroma. In none of the specimens were changes found which were diagnostic of trachoma. The histological appearance was that of chronic inflammation.

DISCUSSION

In spite of several obvious limitations inherent in the present report, certain observations deserved emphasis. Adenovirus types 16 and 17, and strain "931" are pathogenic for man, producing clinical manifestations that may be described as acute follicular conjunctivitis. However, due to severe corneal scarring, it is not known whether the corneae may also be involved. The complete absence of sore throat, fever and preauricular lymphadenopathy in these 3 cases suggested that the infection remained localized; this is in contrast to those described by Bell *et al.*[2] and Jawetz[6] for several other adenoviruses isolated in the United States.

There were some similarities in the clinical manifestations of cases 4 and 5, infected with conjunctival scrapings from local cases of trachoma, and cases 1, 2 and 3, at least during the acute phase of the disease. Of interest is the failure to demonstrate inclusions or elementary bodies in the conjunctival smears taken at regular intervals during the first month. Since after the first month the patients lived in institutions, where trachoma is endemic, and were seen only at intervals of 1 to 6 months, one cannot be certain that subsequent developments are definitely related to the experimental infection. However, the absence of scar formation in these patients about 2 years later is worth noting, especially for cases 4 and 5.

After the completion of the present study, it was noted that the serum used for cell cultivation contained inhibitors for adenovirus type 3 and poliovirus type 1.[4] The paucity of virus reisolation in cases 2 and 3 may possibly be due to such nonspecific viral inhibitors. Since serum from one single horse was used for all studies reported in the present and preceding paper[1] and the level of viral inhibitor in the horse serum remained constant when tested at intervals between 1955 and 1957,[4] viral inhibitors for these adenoviruses, if present, would similarly affect the results in both studies. Thus, the paucity of virus reisolation in cases 2 and 3 increases the significance of the 6 per cent positive isolations reported in the preceding paper.[1]

Similarities in the histological pictures of biopsical materials taken during the acute phase also deserve attention.

SUMMARY

Five blind children were experimentally infected with adenoviruses type 16, 17 and strain "931," and with conjunctival scrapings from 2 cases of trachoma. The clinical signs and symptoms and the course of illness were compatible with those of acute follicular conjunctivitis. Viruses were reisolated in 2 of the 3 cases infected by the adenoviruses while immunological response was detected in all 3 cases. Histological findings of biopsied materials taken during the acute inflammatory stage were similar for all 5 cases.

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THE PREVALENCE OF TOXOPLASMOSIS IN WILD AND DOMESTICATED ANIMALS OF THE MEMPHIS REGION

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During the past seven years a great many animals of various species have been examined serologically or parasitologically by this laboratory for evidence of natural *Toxoplasma* infection. The results of some of these investigations have been published separately (at least in part), but others have remained unpublished. In all, we have isolated organisms from eight species of mammals and birds and have made serologic studies of other species. This paper summarizes all of our studies and presents a picture of the prevalence of *Toxoplasma* infection in animals of this region.

METHODS

Our methods have varied somewhat as our knowledge of *Toxoplasma* has increased. For the serologic studies the dye test was used essentially as described by Sabin and Feldman[13] and Feldman.[4] Blood was taken from animals without anticoagulant and the test run on the serum after inactivation for 30 minutes at 56°C. For most studies 4-fold dilutions were used. In the case of some very small animals and in certain studies low dilutions (undiluted, 1:4) were not used.

For the parasitological studies, brain tissue was used most frequently. Suspensions in saline were inoculated into uninfected laboratory animals, and in almost all cases laboratory mice of the N.I.H. general purpose strain were used. When guinea pigs were employed as in the case of some of the rat studies, two animals were used in each case to minimize the danger of confusing induced and spontaneous infections.

Suspensions of tissue were injected intraperitoneally and recipient animals were observed for signs of infection. Three animal passages were used in most of the studies. In studies involving single passages, the dye testing of surviving animals was usually used to detect asymptomatic infections. Due to slight variation in technique, the results of the various parasitologicla studies are not strictly comparable, and the studies must be individually evaluated.

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OBSERVATIONS

The studies made by this laboratory on various domesticated and wild animals of the Memphis region are summarized in Table 1. This table gives the species involved and the origin of each sample. The paragraphs which follow add information which may aid in the interpretation of the results.

The cat. The cat has been found infected with toxoplasmosis frequently in Memphis as reported in another paper.[10] The present paper presents the same as well as additional information on this species.

Rates for the cats both serological and parasitological were the highest seen in any species, unless significance be attributed to the low titer positive tests in some of the ungulates. The sampling shows that high prevalence rates persisted throughout the 18-month period of study and did not represent a temporary epizootic condition. The cats from the slum areas in Memphis had a distinctly higher infection rate than cats from the pound. It is likely that this was due at least in part to inclusion in the pound sample of a larger proportion of young cats. Unfortunately, we did not obtain an adequate sample of pet cats kept within houses, the urban sample being almost entirely of semiferal animals. The pound sample undoubtedly included pets but these could not be distinguished with certainty. That toxoplasmosis does occur also in pet animals with their different environment is shown by the frequency with which the clinical infection has been seen by some workers.[11] Our sample is also deficient in not including cats from a rural environment.

The urban cat sample was from locations best described as slums. Somewhat over half the sample was taken in the vicinity of a proved case of human toxoplasmosis, but infection was no more frequent in that part of the sample than in the remainder which came from a similar environment several miles away.

TABLE 1

Summary of serological and parasitological studies of toxoplasmosis in animals of the region of Memphis, Tennessee

	Dye test studies									Parasitological studies			
	No. tested	Negative	Undiluted	1:4	1:16	1:64	1:256 and over	% Positive all titers	% Positive 1:16 and over	No. tested	No. found infected	% Infected	Reference if previously published in whole or in part
Pet animals													
House cat													
Memphis pound, early 1956	102	41	10	6	21	20	4	60	44	102	22	22	Jones, Eyles and Gibson, 1957
Memphis pound, 1956-1957	116	72	(<1:16)	—	20	20	4	—	40	27*	17	63	
Urban slum area, 1956	64	10	3	2	18	19	12	84	77	64	17	27	Gibson and Eyles, 1957
Dog													
Memphis pound, 1952–1953	137	73	33	8	11	6	4	45	15	137	0	0	
Memphis pound, 1953–1954	663	557	(<1:16)	—	52	41	13	—	16	54*	2	4	
Urban slum area, 1956	9	6	0	0	2	1	0	33	33	9	1	11	
Domesticated birds													
Chicken													
Rural, Fayette Co., Tenn., 1953	50	45	4	1	0	0	0	10	0	50	0	0	
Urban slum area, 1956	6	3	3	0	0	0	0	50		6	3	50	Gibson and Eyles, 1957
Semirural, Sardis, Miss., 1957	10	10	0	0	0	0	0	0	0	10	0	0	
White Peking Duck													
Urban slum area, 1956	2	0	1	1	0	0	0	100	0	3	2	67	Gibson and Eyles, 1957
Pigeon													
Urban slum area, 1956	15	14	0	0	1	0	0	7	7	16	1	6	Gibson and Eyles, 1957
Domesticated animals													
Swine													
Memphis abattoir, 1952	50	5	11	22	9	0	2	90	22		†		
Herd, Shelby Co., penal farm, 1953	98	39	11	29	18	1	0	59	19	99	0	0	
Custom slaughterhouse, Memphis, 1957	30	5	10	7	7	1	0	83	26	30	1	3	
Cow													
Memphis abattoir, 1952	50	0	40	10	0	0	0	100	0		†		
Horse													
Memphis abattoir, 1952	50	33	8	7	2	0	0	34	4		†		
Mule													
Memphis abattoir, 1952	50	33	6	11	0	0	0	34	0		†		
Sheep (lambs)													
Memphis abattoir, 1952	29	0	7	15	7	0	0	100	24		†		
Wild rodents													
Norway rats (*Rattus norvegicus*)													
Laboratory albino strain	74	73	1	0	0	0	0	1	0		†		
Urban Memphis, 1950	100	80	12	0	6	0	2	20	8	190	6	3	Eyles, 1952
Rural, Fayette Co., Tenn., 1953	4	4	0	0	0	0	0	0	0	11	0	0	
Semirural, Sardis, Miss., 1957	25	25	0	0	0	0	0	0	0	25	0	0	

TABLE 1—*Continued*

	Dye test studies									Parasitological studies			
	No. tested	Negative	Undiluted	1:4	1:16	1:64	1:256 and over	% Positive all titers	% Positive 1:16 and over	No. tested	No. found infected	% Infected	Reference if previously published in whole or in part
House mouse (*Mus musculus*)													
Laboratory albino strain	74	74	0	0	0	0	0	0	0		†		
Rural, Fayette Co., Tenn., 1953	†									54	0	0	
Urban, slum area, 1956–1957	51	49	0	0	1	0	1	4	4	220	9	4	Gibson and Eyles, 1957
Semirural, Sardis, Miss., 1957	2	2	0	0	0	0	0	0	0	7	0	0	
Cottontail rabbit (*Sylvilagus floridanus*)													
Rural, Fayette Co., Tenn.	9	2	1	5	1	0	0	78	11	9	0	0	
Fox squirrel (*Sciurus niger*)													
Rural, Arkansas, 1952	19	19	0	0	0	0	0	0	0	19	0	0	
Wild birds													
House sparrow (*Passer domesticus*)													
Rural, Shelby Co., Tenn., 1953	36	‡36	0	0	0	0	0	0	0	36	0	0	
Urban, slum area, 1956	9	9	0	0	0	0	0	0	0	9	0	0	
White-crowned sparrow (*Zonotrichia leucophrys*)													
Rural, Shelby Co., Tenn., 1953	5	‡5	0	0	0	0	0	0	0	6	0	0	
Cardinal (*Richmondena cardinalis*)													
Urban, Slum area, 1956	7	7	0	0	0	0	0	0	0	13	0	0	
Mourning Dove (*Zenaidura carolinensis*)													
Rural, Sardis, Miss., 1957	4	4	0	0	0	0	0	0	0	17	0	0	
Turkey Vulture (*Cathartes aura*)													
Rural, Shelby Co., Tenn., 1952	9	9	0	0	0	0	0	0	0	9	0	0	
Black Vulture (*Coragyps atratus*)													
Rural, Shelby Co., Tenn., 1952	21	21	0	0	0	0	0	0	0	21	0	0	

* Selected sample, only animals with positive dye tests used.

† Not done.

‡ Undiluted tests not done on some of these due to small quantity of blood.

The dog. Memphis dogs are frequently infected with *Toxoplasma* judging by the dye test results, as each of the samples showed from 15 to 33 per cent positive at dilutions of 1:16 or greater. Even so we were able to recover parasites only infrequently, even when dye test positive dogs were selected for study.

High titer positives were about half as frequent in Memphis pound dogs as in cats from the same source, and parasites were recovered very much less frequently. This is not in accord with the observation of Feldman and Miller[5] who found about the same dye test prevalence in the two species in Syracuse, New York, but we believe

our findings indicate a real difference in our locality at least for the time period studied. The dog findings are discussed in greater detail in a separate paper from this laboratory.[7]

The chicken. The dye test indicated that the chicken rarely has detectable dye test antibodies, but this observation is deceiving as experimental studies by us[9] and others[1] have shown that the dye test response of this species is transient and feeble. The parasitological studies indicated that infection is rare or absent in rural flocks, but present in at least some urban flocks. Unfortunately the only urban sample was taken from the environs of a case of human toxoplasmosis.[6] Whether the same high prevalence would have been found elsewhere in the city is problematical, but certainly in the case of the cat similar prevalence did prevail elsewhere.

The white peking duck. The finding of two infected birds added another species to those known to be naturally infected with *Toxoplasma*. As in the case of the urban chickens, the duck sample consisting of only three birds is inadequate and unrepresentative.

The pigeon. The pigeon has been found infected previously in a number of localities, so our finding of the parasite in one of 16 birds was not unexpected. Despite the small size of our sample, we were actually surprised that we did not find a higher prevalence.

Swine. Much attention has been given swine as a possible source of human toxoplasmosis due to the finding of the parasite in this animal by Farrell *et al.*,[3] and the experimental work of Weinman and Chandler.[14, 15] More recently Jacobs[9] reported a high prevalence of infection as measured by the recovery of organisms from hog diaphragm muscle.

Our serological results show a titer distribution such as might be expected in a host which is frequently naturally infected. The high number of low titer positives may represent antibody passively transferred from the sows as many pigs were rather young when slaughtered. Even discounting the low titer positives, a significant proportion were positive at 1:16 or greater dilution.

Parasitological studies have not shown a correspondingly high degree of infection; in fact, we have recovered only a single strain from 129 swine studied. In all of our work brain tissue was principally used for inoculation procedures. Perhaps the discrepancy between prevalences found by us and that reported by Jacobs is due to the persistence of parasites to a greater degree in muscle than in brain tissue. In spite of this possibility, we believe it is much more likely that we were measuring a geographic difference such as has been shown in trichinosis.

Other ungulates. Only serological studies have been made of the other domesticated ungulates. Fifty cattle were all dye test positive but only at the lowest titers. We believe it highly doubtful that the positive responses were due to toxoplasmosis although there is little experimentally derived information on the response of cattle to infection. Sanger *et al.*[16] infected cows experimentally but presented no dye test data in their account of the work.

Feldman and Miller[5] reported a similar high prevalence of low titers in one herd studied by them, but did not observe this phenomenon in other herds. Our sample was not of a single herd but was representative of cattle coming to slaughter from the Memphis region.

Horses and mules, which were mostly very old animals, were nearly all negative to the dye test and only a few showed titers as high as 1:16. Sheep on the other hand showed a high prevalence of positive reactions with a significant number at the 1:16 level. The sheep in question were mostly lambs believed to be about six months old so the possibility of passively transferred antibody must be considered.

The Norway rat. Seventy-four albino laboratory rats were dye tested and all were negative but one which was positive undiluted. This is further evidence of the infrequency of infection in laboratory reared rats. Wild rats gave varying results. The urban sample, which has been previously reported,[2] showed a dye test prevalence of about 20 per cent and a parasite prevalence of about three per cent. Contrasting with this, 29 rural or semirural rats were all negative both serologically and parasitologically.

The house mouse. Dye tests of 74 laboratory albino mice of the N.I.H. general purpose strain were all negative. Numerous experiments have shown that our laboratory mice are universally susceptible and that spontaneous infection rarely if ever takes place under laboratory conditions.

Sufficient blood for dye testing could only rarely be obtained from trapped house mice. The samples obtained were mostly negative and it is of

interest that strains were recovered from both of the wild mice which had positive tests. Recovery of parasites was also infrequent and six of the nine infections demonstrated came from the residence of a proved human case.

Other wild rodents. No infections were demonstrable in 19 fox squirrels and dye tests of the squirrels were all negative. No infections were found parasitologically, but seven of nine cottontail rabbits were dye test positive, mostly at low titers. A single gray squirrel (*Sciurus carolinensis*), cotton rat (*Sigmodon hispidus*) and muskrat (*Fiber zibethicus*) were all negative parasitologically but were not included in Table 1.

Wild birds. All of the wild birds tested were negative both serologically and parasitologically. In addition to the species shown in Table 1, a single starling (*Sturnus vulgaris*), mockingbird (*Mimus polyglottos*), brown trasher (*Toxostoma rufum*) and two blue jays (*Cyanocitta cristata*) were also found uninfected.

The negative results of the testing of 30 vultures were of particular interest as these species consume meat. Of course, carrion is usually consumed but sick animals are frequently eaten shortly after death and laboratory work has shown that parasites remain viable in carcasses up to several days.

DISCUSSION

The ultimate objective of much current epidemiological work on toxoplasmosis is to determine how man and animals become infected. Certainly there must be an interrelationship between infection in the various hosts and most likely some of the animals associated with man pass the infection on to him. A picture of the prevalence of the parasite in the various hosts is a prerequisite to a study of the epidemiology of the disease.

The data presented, involving the study of more than 2,300 animals, gives a picture of the distribution of *Toxoplasma* infection in the Memphis region. Some noteworthy facts stand out. For example, nearly all of the animals closely associated with man, which were tested parasitologically, were shown to be infected at least occasionally. These include the cat and dog; the chicken, duck and pigeon; the rat and mouse; and the pig. On the other hand, no infections whatsoever were demonstrated parasitologically in wild animals not associated with man, although, admittedly, fewer were tested. Could it be that *Toxoplasma* infection is characteristically an infection associated with man's environment? This hypothesis requires further testing.

Granting that man does obtain infection from animals associated with him and that ecologically toxoplasmosis may be an infection primarily of domestic and semidomestic animals, no clear link between infection of the various species has become evident. Certainly, the possibility of infection by the consumption of infected tissues would be a possibility. Transmission by consumption of pseudocysts was easily accomplished in the laboratory in some species by us and by others.

The infections found in chickens, ducks and pigeons are not entirely compatible with infection by the consumption of uncooked meat, although it must be conceded that these species are omnivorous and may sometimes eat scraps of animal tissue.

The common occurrence of the infection in the pet animals, the dog and the cat, certainly points toward the possibility of infection of man by direct contact. Even though direct infection is seldom observed in the laboratory, Olafson and Monlux[12] did describe transmission between dogs in a kennel and it is known that in experimental and perhaps natural infections viable organisms occur in nasal secretions and pulmonary exudates. Some studies have called attention to relationships between infections in man and in pet animals and this possible mode of transmission must continue to receive consideration.

An interesting and perhaps significant observation of the present study, which has been previously discussed,[6] is the occurrence of a number of infected mice in a toxoplasmosis household, contrasted with rare mouse infection in other nearby households. Similar investigations in the future may determine if this was a casual occurrence or if a relationship between infection in the mouse and man existed.

Feldman and Miller[5] have pointed out that the same animal species may present a very different serological prevalence picture from different localities, just as human populations vary. The same limitation is necessary when considering parasitological findings. Thus our results cannot be taken to represent the various species as a whole but merely the groups which we sampled. Even so, the various studies made about the

world have shown qualitatively similar findings; that is, that infection is frequent in dogs and cats, and not uncommon in rats, chickens, pigeons, and swine. Our paper contributes additional information on mice and ducks.

A limitation to the interpretation of the serologic results of the study is imposed by our ignorance of the nature of the dye test response in some of the animals. We do know that the dog, cat, rat, rabbit, mouse, pig and perhaps the pigeon react much as does man, but we also know that the chicken reacts only feebly, and we have no information concerning the reaction of cattle, horse, sheep, wild birds, ducks or squirrels. This being so we can only speculate on the significance of the negative and low titer positives observed in these hosts, and discussion of the specificity of the findings is not warranted.

SUMMARY

A total of over 2,300 animals principally of 20 species of pet animals, domesticated animals, wild rodents and birds were studied by means of the dye test for toxoplasmosis or by parasitological methods or both. The findings were as follows:

1. Memphis cats were very frequently found infected with *Toxoplasma* judging by both the dye test and parasitological study. Rates were the highest for any species studied.

2. Dogs showed a significant number of serologic positives and infections were demonstrable infrequently.

3. Infections were also found in chickens, a pigeon and domesticated ducks but samples were insufficient to estimate general prevalence.

4. A single swine infection was found, but serological results indicated infection was fairly frequent. Serological results on cattle, horses, mules and sheep were presented but lack of experimental knowledge precluded interpretation.

5. Rats were found infected occasionally as were house mice. Six infected mice were found in a household in which a human case of toxoplasmosis had occurred.

6. No infections were found in wild rodents or wild birds nor was there serological evidence of infection except perhaps in cottontail rabbits.

7. The results of the study are discussed and evaluated as related to the epidemiology of the disease.

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HISTOLOGIC INVESTIGATION OF MUSCLE TISSUE IN KWASHIORKOR[1]

MARC VINCENT[2] AND MARIE-ANGE RADERMECKER[3]

INTRODUCTION

At the 1953 Conference on Protein Nutrition held under the auspices of the F.A.O., W.H.O., and the Josiah Macy, Jr. Foundation in Jamaica, B.W.I., it became clear to all investigators that there had been very few studies of muscle tissue in kwashiorkor and similar diseases even though muscle is the principal protein reservoir of the living organism.

Kwashiorkor, a disease of protein malnutrition, is characterized by a variety of features; usually, weight loss, edema, diarrhea, atrophy of the muscles, dyspigmentation of the hair, dermatosis, anemia, and fatty infiltration of the liver. It is most often seen in children shortly after weaning. The disorder has been given the name it had for centuries among the Ga tribe of Ghana, where it came to the attention of early investigators.

Although the name was introduced in European medical literature about 30 years ago, kwashiorkor itself has been studied systematically only during the past 10 years. No article on the state of muscle tissue in cases of malnutrition or cachexia was published before World War II.

In 1869 Ollivier was the first writer to discuss the question of the effect of diet on muscle tissue. He was followed by Hayem in 1875 and Bompar in 1887. All three were primarily concerned with the lesions found in the adult.

In 1902 Durante, writing in Cornil and Ranvier's *Treatise*, described in detail the development of muscle tissue in the newborn. He felt that pathological processes involving muscles resulted in an involution toward an embryonal state of an essentially protoplasmic tissue. He concluded that the sarcoplasm should receive the concentrated attention of the pathologist. He also pointed out that the proliferation of interstitial tissue was probably the result of an irritative phenomenon caused by products of degeneration. Bramwell and Muir described muscle atrophy in sprue in 1907, and in 1913 Glansburg and Topley attempted to show that the characteristic alteration of nutritional muscular atrophy consisted in a variable diminution of the diameter of the muscle fibers.

The first article to deal with muscle tissue in kwashiorkor appears to have been one by Bablet and Normet in 1937. These authors described a severe degeneration of myocardial fibers with lysis, loss of architecture, disappearance of tonofibrils and striations, hypertrophy of the nuclei, and vacuolization of the fibers. The interstitial lesions comprised diffuse collections of monocytes in the interfascicular spaces and occasionally in the fascicles themselves. These authors also described a rather mild leukocytic infiltration of the pericardium without giant cells.

In their monograph on kwashiorkor published in 1954, Trowell, Davies and Dean cited Bablet and Normet's findings and added that in some cases they had found a loss of subendocardial muscle fibers in the ventricles, particularly the left ventricle. They also noted small interstitial collections of lymphocytes and macrophages. The most striking lesion, however, was an interstitial edema with fatty degeneration or vacuolization but with simple thinning and atrophy of the muscle fibers. These two are the only descriptions of the muscle changes in kwashiorkor that we have been able to find in the literature.

Waterlow (1955, 1956), taking a biochemical approach to the problem, measured the nitrogen:desoxyribonucleic acid ratio in muscle and liver. This ratio, which is normally about 80, falls to 40 or 50 in kwashiorkor. Since the ratio falls more abruptly and severely in muscle tissue than in liver, Waterlow concluded that in patients with this disease the main site of tissue protein depletion occurs in muscle. He stated that the amount of desoxyribonucleic acid found in nuclei is constant and that since it is not affected by edema, it would be a more reliable indicator than the amount of nitrogen per unit weight of tissue. However, cellular

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multiplication, which is such a characteristic reaction of pathologic states of muscle, would lower the nitrogen:desoxyribonucleic acid ratio by increasing the latter.

It is perhaps to be expected that in kwashiorkor the organism would call upon its tissue protein reserve in order to maintain the level of serum proteins, and the hyperplasia of the adrenal cortex actually does result in more effective utilization of tissue proteins. Kwashiorkor is a condition in which many enzymatic and metabolic processes are affected. It is possible that in this disease utilization of tissue protein proceeds along lines other than the normal mechanisms. It is also possible that because of some enzymatic dysfunction there might be an accumulation of intermediary metabolites which, in certain concentrations, might become toxic. In such an eventuality, morphologic lesions might appear. At first these might be reversible but in time they would become irreversible. Thus, the duration and the severity of the disorder would result in a gradation of pathologic changes.

MATERIAL AND METHODS

Seventeen Bantu children ranging in age from 9 months to 10 years were under observation by one of us (MV) on the Pediatric Service of the Hospital Prince Regent Charles in Usumbura, Ruanda-Urundi. The admission diagnosis of our cases was kwashiorkor, although this diagnosis was not substantiated in every instance. After the final diagnoses were made, we were able to compare the specimens of muscle from well documented and well established cases of the disease, with those obtained from children with cachexia due to various other conditions.

Detailed physical examinations, hematologic studies and investigation of serum proteins were carried out on all these patients. Total serum protein was determined by the copper sulfate method of Van Slyke, and paper electrophoresis by the method of Sonnet and Rhodain (1952). A liver biopsy was attempted in each case but was unsuccessful in a number of instances because of technical factors. Muscle tissue was obtained from the deltoid in Cases 3 to 11 and from the trapezius in the other patients. The muscle specimen was fixed in 8 per cent Formalin, embedded in paraffin and stained with hematoxylin eosin. A van Gieson stain was also used on some of the cases.

In addition to the proven cases of kwashiorkor, we have included two atypical ones (Cases 19 and 20). These two patients were diagnosed as suffering from protein deficiency with edema. According to Platt (1958), protein malnutrition must be appreciated not strictly in terms of intake, but also in relation to the various needs and stresses of the organism. Thus, even though the serum proteins in Case 19 were normal, the widespread edema and fatty infiltration of the liver suggested that protein metabolism in that patient was far from normal We felt that Case 20 was an example of very early kwashiorkor. In this case there was only mild leg edema and normal tissue; none the less, the clinical history was typical and the serum protein levels were well below normal.

PATHOLOGIC FINDINGS

Analysis of the materials obtained by muscle biopsy allowed us to distinguish a gradation of severity of the muscular lesions. The lesion in the least advanced case consisted simply of a slight increase in the number of nuclei in the sarcolemma. It is well known that nuclei are more abundant in young children, but in our cases this number was definitely larger than that found in muscle tissue obtained from controls of the same age (Figs. 1 and 2). The next lesion showed an increase in size of the endothelial nuclei of capillaries crossing muscle fibers. These nuclei were much more distinct and larger than normal and were especially well seen where a capillary crossed a fiber containing an increased number of pale nuclei (Fig. 3). As the disease progressed, small collections of lymphocytes appeared around these capillaries and, in an even more severe lesion, small bunches of mesenchymal fibrils could be seen along these vessels. These very fine fibers could be seen quite easily in some cases (Fig. 4). Concurrently with the increase in number and size of nuclei, there was a mild and thin intermysial connective tissue reaction in the vicinity of the capillaries. A few lymphocytes, and rare histiocytes were also found in the same areas.

In all of our cases the alterations of the muscle fibers themselves occurred only in isolated, widely scattered areas. These often consisted of loss of striation with an increase in number of

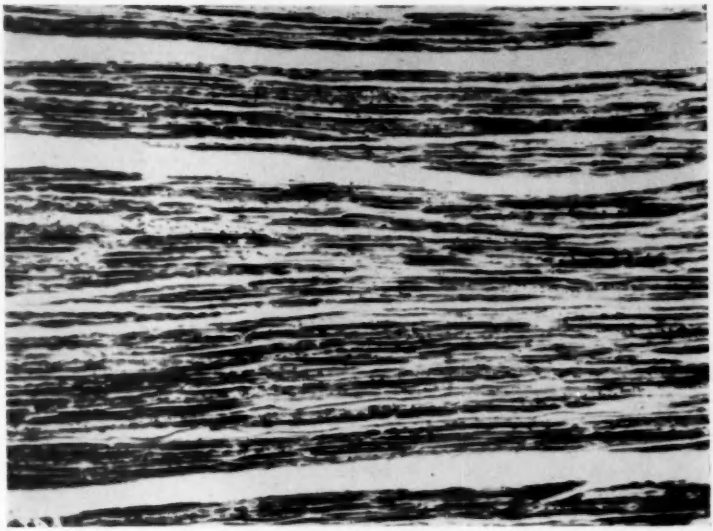

FIG. 1. Case 7: Overall view of edematous distention of the intermysium. Note the increase in the number of both pale and dark nuclei and a few shredded fibers. (paraffin, van Gieson)

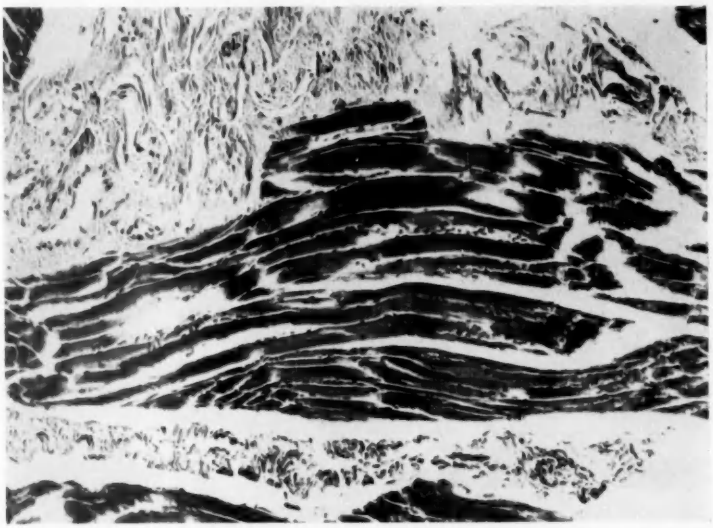

FIG. 2. Case 21: Slightly more pronounced increase in intermysial nuclei and variation in fiber caliber. (paraffin, van Gieson)

dark nuclei which seem to be arranged in rows. A few cells might appear inside the fiber which then became eosinophilic to a marked degree. In more severe lesions the sarcoplasm might become fragmented or even disappear, leaving behind only a few remnants of acidophilic sarcoplasm surrounded by nuclei. (Fig. 5). As a rule, the blood vessels did not show any changes

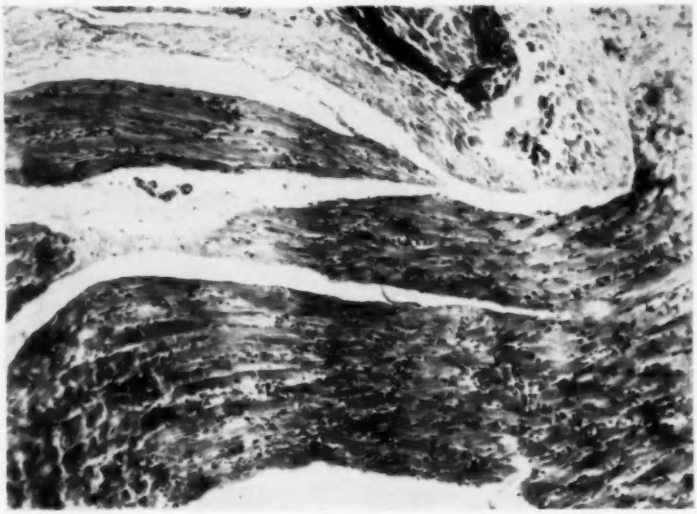

FIG. 3. Case 18: The increase in dark nuclei is even more marked, especially around certain fibers and capillaries crossing them. (paraffin, van Gieson)

FIG. 4. Case 18: Higher magnification showing the bunches of lymphocytes and fibroblasts where capillaries cross muscle fibers. (paraffin, van Gieson)

in their walls except for occasional edematous distention of the adventitial space. The fine, intramuscular, nervous rami might also appear to be distended by edema and occasionally there was an increase of Schwann cells and even of fibroblasts in their vicinity. These latter changes, however, should not be held responsible for muscle changes of neural origin.

The most impressive finding in all of our preparations was the variation in caliber of the

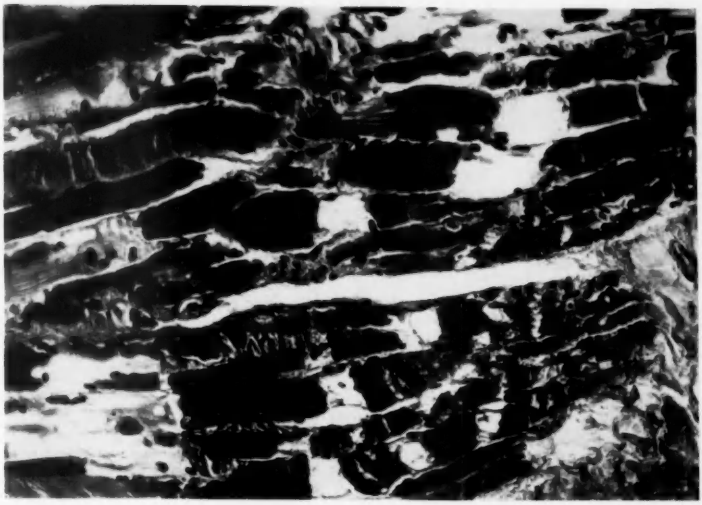

FIG. 5. Case 18: Fragmentation and resorption of isolated fibers. (paraffin, van Gieson)

TABLE 1

Findings in cases of Kwashiorkor

Patient and sex	Case No.	Age (*yr, mo*)	Duration of malnutrition	Weight (*kilos*)	Estimated degree of muscular atrophy	Edema		Skin		Hair discoloration	EKG changes	Serum proteins gm/liter	Alb. (%)	Liver biopsy	Severe or mild case
						limbs	face	CPS*	MS*						
Leo ♀	4	8, 0	several yr.	11.800	+++	+	++	+	+	++	++	38	18	–	S
Nda ♀	7	2, 6	?	7.900	+	++	++	+	+	++	0	49	32	–	S
Kab ♂	8	3, 0	1 mo.	6.850	++	+	+	0†	0	?	++	55	43	–	S
Jum ♂	11	1, 10	several mo.	7.500	++	++	++	+	+	++	0	39	35	–	S
Nze ♀	18	1, 11	several mo.	7.400	++	++	++	0	0	++	++	56	19	Fatty	S
Nge ♂	21	3, 1	1 mo.	8.200	++	++	++	0	+	?	0	39	20	Fatty	S
Bay ♂	22	1, 0	1 mo.	7.050	+	++	+	0	+	+	0	43	20	Fatty	S
Riz ♀	23	3, 0	1 yr.	9.100	++	++	+	++	+	++	–‡	43	17	Fatty	S
Kas ♀	25	1, 3	15 days	6.900	+	+	+	0	+	0	++	38	48	Fatty	S
Nku ♀	19	1, 2	15 days	8.200	0	++	++	0	0	+	–	52.5	47	Fatty	M
Niy ♂	20	0, 11	15 days	8.500	0	+	0	0	0	0	0	47	32	N	M

* CPS = "crazy pavement" skin; MS = mosaic skin.

† 0 = examination was negative

‡ – = specimen unsatisfactory or lost

muscle fibers in different fields. Many were considerably reduced in size. In addition, another striking change was the edematous infiltration of the interfascicular septa.

This gradation of pathologic changes permits us to separate our cases of kwashiorkor into three groups according to severity: the first group, consisting of cases 20, 22 and 25, shows only mild alterations; the second group, cases 9, 10, 11, 21, and 23, has more definite lesions;

TABLE 2

*Findings in cachexias due to causes other than kwashiorkor**

Patient and sex	Case No.	Age (*yr, mo*)	Duration of disease	Weight (*kilos*)	Estimated degree of muscular atrophy	Edema		Skin		Hair discoloration	EKG changes	Serum proteins gm/liter	Alb. (%)	Liver biopsy	Diagnostic
						limbs	face	CPS	MS						
Muk ♂	3	8, 0	4 mo.	20	+	0	+	0	0	0	++	54	40	–	Hepatic cirrhosis, typhoid fever
Thé ♀	5	5, 0	?	8.200	+	0	+	0	0	0	–	50	26	6	lympho-sarcoma
Mat ♂	6	1, 4	1 mo.	7.600	0	0	0	0	0	+	0	68	59	–	ankyloso-miasis
Tat ♀	9	1, 0	1 mo.	8.400	0	0	0	0	0	0	++	42	44	–	noma
Amu ♂	10	10, 2	3 yr.	16	+	0	0	0	0	0	+	51	19.5	–	hepatic cirrhosis
Nda ♂	24	4, 10	1 mo.	12.700	+	0	0	0	0	0	+	53	41	No fatty infiltr. Miliary tuberc.	granulie tubercu-losi

* See footnotes to Table 1.

while the third group, cases 7, 8 and 18, shows more severe pathologic changes.

In one of our cases we found lesions of advanced fibrous organization which do not fit in at all with the characteristic lesions found in all our other patients. We also studied muscle tissue obtained from cases of cachexia due to causes other than kwashiorkor. In those patients we frequently noted an increase in the number of the clear sarcolemmal nuclei and, often less, an edematous infiltration of the septa and interfascicular membranes with an early eosinophilia of the muscle fibers. In only one instance (Case 5) did we observe rare, isolated fibers exhibiting degenerative lesions with pericapillary lymphocytic reaction in a few septa.

DISCUSSION

In kwashiorkor, as in other conditions resulting in cachexia, the order of reaction of muscle tissue is as follows: first the sarcolemma, then the intermysial tissue, and finally the pericapillary areas. This reaction, which has both an infiltrative and fibroblastic component, is more marked and definite in kwashiorkor while barely recognizable in other cachectic states. It is best seen in areas where a capillary crosses a muscle fiber. Lesions involving the muscle fiber itself are exceptional in cachectic states while they are seen in isolated fibers in severe cases of kwashiorkor. Blood vessels and nerve fibers may exhibit the same changes as the setae. Striations, on the other hand, seem to be quite stable and, in cachexias other than kwashiorkor, the fibers appear to retain their normal functional integrity.

These findings are indeed interesting in light of the biochemical investigations carried out by Dreyfus, Schapira and Kruh (1957). These investigators injected radioactive glycocol C^{14} in rats and demonstrated that the half-life of sarcolemmal soluble proteins was of the order of 15 days with a rapid loss of radioactivity while the insoluble protein (actynomyosin) of the contractile fibers exhibited a stable radioactivity for a period of 40 days. This is probably the reason why the radioactivity of muscle remains elevated for a much longer period than that of liver. Dreyfus, Schapira and Kruh believe that the fibrillary proteins do not draw upon a common pool of amino acids. The metabolism of the soluble proteins might be the subject of fruitful investigations in the future.

SUMMARY AND CONCLUSIONS

1. Muscle biopsies in children with kwashiorkor reveal an increase in the number of both pale and dark nuclei of the sarcolemma, a pericapillary infiltration of the interfascicular vessels, edema, and, in more advanced cases, a fine network of connective tissue fibers in the intermysial space. This intermysial sclerosis may include some infiltrative elements (lymphocytes, more rarely histiocytes). The caliber of the muscle fibers and of the fascicles is frequently reduced. Degenerated muscle fibers occur at random. No evidence of burgeoning can be seen in any of the affected muscle fibers. The adventitial spaces of blood vessels and the small nerve fibers may show signs of edema. These findings are based upon the study of 11 cases of kwashiorkor.

2. In seven cases of cachexia due to causes other than kwashiorkor, muscle tissue showed a slight increase in the number of the pale nuclei, some septal edema, slight eosinophilia of a few muscle fibers and, rarely, a few degenerated fibers.

3. In both instances, the lesions are of the same type but they are uniformly more severe in the patients with kwashiorkor.

4. The muscle fibers themselves would appear to be the most resistant element of the fascicle. These are involved only when the edema and the fibrous changes are advanced. This occurs only in cases of kwashiorkor presenting the full clinical picture.

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HUMAN INTESTINAL HELMINTHS OBTAINED FROM AUTOPSIES IN DJAKARTA, INDONESIA

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Although intestinal worm infections in man are common in Indonesia, accurate parasitological surveys are few. The present report is based upon an examination of 664 autopsies performed in Djakarta in the years 1952–1955.

MATERIALS AND METHOD

The material consisted of 595 forensic autopsies and 69 clinical autopsies. The forensic autopsies were performed on persons who died from accidents, suicide, murder and included persons who were found dead in the streets. The clinical cases consisted of persons who died of illness in the General Central Hospital and whose bodies were not claimed by their relatives. The age of many corpses was not known and an estimated age was used in these cases. There were 518 males and 146 females. The residency of 589 cases was known. Most of them lived in the city itself while a small number came from the rural or semirural surroundings of Djakarta. There were 586 Indonesians, 69 Chinese, most of them born in Indonesia, and 9 European. As far as could be judged from their occupation and place of residence most of the Indonesians were of the lower socioeconomic class, while most of the Chinese and Eureopean came from the middle socioeconomic level.

The worms were collected as follows. After the whole gut was removed from the body, water was passed through its oral end and collected in large bowls from the aboral end. This water which contained most of the worms and the whole intestinal content, was strained through two wire sieves with apertures of 1.5 mm and 0.4 mm, respectively, one above the other. The worms were collected from the sieves. The intestine was then opened throughout its whole length to collect the worms which were still attached to the intestinal wall.

This method enabled us to collect all of the larger worms but was inadequate to obtain the smaller worms such as *Heterophyid* flukes, *Strongyloides stercoralis* and others.

RESULTS

The following worms were found: *Necator americanus*, *Ancylostoma duodenale*, *Ancylostoma ceylanicum*, *Ancylostoma caninum*, *Trichuris trichiura*, *Ascaris lumbricoides*, *Oxyuris vermicularis* and occasionally some of the smaller worms such as *Strongyloides stercoralis* and *Trichostrongylus* species.

Ninety-eight per cent of the cases (651 cases) showed infection with one or more species of worms. The frequency of worm infection in Indonesian was 99% (583 cases positive), and in Chinese 87% (60 cases positive). Most of the infected cases had more than one species of worms but in 67 Indonesians and 5 Chinese infection with a single worm speices only was found.

Hookworms

Hookworms were most frequently found of all the intestinal worms, with an incidence of 85.5% and an average worm load of 60.8. Males and females were about equally infected, but the average worm load in males was significantly higher than in females (Table 1); 74.2% (330 cases) of the infected males had less than 100 worms, 14.8% (66 persons) had between 101 and 200 worms, and 11% (49 persons) had more than 200 worms. The highest worm burden found in man was 877. Ninety-two per cent (113 cases) of the infected females harbored less than 100 worms, 4% (5 cases) had between 101 and 200 worms and 4% (5 cases) had more than 200 worms. The highest worm load was 335.

The incidence of hookworm infection was high for all age groups, with the peak lying in the age group 16 to 25 years (Table 2). The highest average worm load was found in the age group 41 to 60 years. Forty per cent of the preschool-age children were infected; the highest worm load in this group was 106. School-age children showed an incidence of 77% with an average worm load as high as in adults (63). There were among these school-age children 8 cases (15%) with more than 100 worms; the highest worm burden was 443.

TABLE 1

Incidences and average worm loads of intestinal worms obtained from autopsies in Djakarta

	Males (518)*		Females (146)		Total (664)	
	Pos. (%)	Average worm load	Pos. (%)	Average worm load	Pos. (%)	Average worm load
All hookworms	86 (445)*	67.4	84 (123)	37.5	85.5 (568)	60.8
N. americanus	85 (441)	63.9	83 (121)	35.8	85 (562)	57.9
A. duodenale	41 (214)	8.3	38 (56)	3.6	41 (270)	7.3
T. trichiura	84 (434)	12.7	89 (130)	12.7	85 (564)	12.7
A. lumbricoides	56 (290)	5.2	77 (113)	5.5	61 (403)	5.3
O. vermicularis	39 (202)	22.	40 (58)	36.3	39 (260)	25.2

* Number of cases.

TABLE 2

Incidence and average worm loads of intestinal worms arranged according to age

	0–5 years		6–15 years		16–25 years		26–40 years		41–60 years		60–	
	Pos. (%)	Average worm load	Pos. (%)	Average worm load	Pos. (%)	Average worm load	Pos. (%)	Average worm load	Pos. (%)	Average worm load	Pos. (%)	Average worm load
All hookworms	40 (8)*	20	77 (42)	66	90 (166)	62	88 (252)	54	83 (90)	82	85 (10)	38
N. americanus	35 (7)	17	77 (42)	63	90 (166)	58	87 (247)	52	83 (90)	77	85 (10)	35
A. duodenale	5 (1)	45	36 (20)	7	47 (87)	7	41 (116)	6	40 (44)	10	25 (3)	9
T. trichiura	55 (12)	3	85 (47)	20	84 (156)	17	88 (250)	10	83 (90)	11	75 (9)	17
A. lumbricoides	80 (16)	8	67 (37)	14	67 (123)	6	55 (157)	5	58 (63)	5	58 (7)	6
O. vermicularis	45 (9)	14	35 (19)	15	32 (59)	20	41 (116)	12	46 (50)	12	58 (7)	89
Total number of autopsies	20		55		184		285		108		12	

* Number of cases.

Indonesians showed a higher incidence and a higher average worm load then Chinese (Table 3).

N. americanus was most frequently found of all hookworms (84.6%) with an average worm load of 57.9. *A. duodenale* showed a lower incidence (40.7%) and a low average worm load of 7.3. For both species the average worm load was higher in males than in females. Infection with *A. duodenale* was almost always associated with infection with *N. americanus*. In 6 cases only no concomittant *N. americanus* infection was found.

The incidence of *A. duodenale* in Indonesians was higher than in Chinese, but the average worm load was the reverse in the two races (Table 3). In Indonesians a total of 1,768 *A. duodenale* and 31,085 *N. americanus* were found. The per-

TABLE 3

Incidences and average worm loads of intestinal worms arranged according to race

	Indonesians (586)*		Chinese (69)		European (9)	
	Pos. (%)	Average worm load	Pos. (%)	Average worm load	Pos. (%)	Average worm load
All hookworms	89 (524)*	62.7	59 (41)	39	33 (3)	17
N. americanus	89 (519)	59.9	58 (40)	35	33 (3)	17
A. duodenale	43 (252)	7	25 (17)	12	11 (1)	1
T. trichiura	87 (509)	13.3	70 (48)	7	77 (7)	6
A. lumbricoides	64 (373)	6	42 (29)	4	11 (1)	7
O. vermicularis	39 (231)	25.7	36 (25)	14	44 (4)	41

* Number of cases.

centage of ancylostomes to necators was therefore 5.7. In Chinese a total of 210 *A. duodenale* and 1,390 *N. americanus* were found and the percentage of ancylostomes in this group was 15.

A. ceylanicum was found seven times, each time one worm only was recovered. All cases were observed in Indonesians, six times in adults and one time in a boy 10 years of age. There were five female and two male worms. They were carefully examined and special attention was paid to the bursa and inner ventral teeth. All resembled *A. ceylanicum* as described by Biocca.[1]

A. caninum was found once in an Indonesian male, 36 years old. One male worm only was recovered. The diagnosis was based on the presence of three pairs of large ventral teeth in the buccal capsule.

Trichuris trichiura

This worm was found in a high frequency (84.9%) with an average worm load of 12.7. There was no significant difference in incidence in males and females and all age groups showed a high incidence. The preschool-age group showed a low average worm load. Indonesians were more affected than Chinese. The highest worm load found in a person was 152.

Ascaris lumbricoides

The incidence (60.8%) and average worm load (5.3) of *A. lumbricoides* were somewhat lower than for *T. trichiura*. However, in the preschool-age group *T. trichiura* showed a lower frequency and average worm load. The incidence of *A. lumbricoides* was higher in Indonesians than in other races. The largest number of worms found in a person was 60.

Infection with *A. lumbricoides* was usually associated with *T. trichiura*. In five cases only no concomittant *T. trichiura* was observed.

Oxyuris vermicularis

The incidence of *O. vermicularis* was 39.1%, more or less equal in males and females, in different age groups and also in different races. The average worm loads shown in the tables were probably low because many male worms were lost during the process of collection.

DISCUSSION

Djakarta with 2.5 million inhabitants has two types of living quarters. One consists of brick houses built along paved streets and occupied by big shops, offices and homes of the higher and middle socioeconomic class. A sufficient water supply and adequate sanitary conveniences are available. The other type consists of smaller wood and bamboo houses which are often built close together, along narrow paths and back streets; these are the kampongs of Djarkata which are situated next to or often inclosed by blocks of the better homes. In these crowded kampongs without adequate water supply and often without electricity, people, mainly of the lower and partly

also of the middle socioeconomic class, live under poor sanitary conditions. Due to lack of sanitary conveniences many of them make a practice of defecating on the ground, in the ditches, drains, rivers and canals. Not infrequently they bathe their bodies and wash their rice and clothes in the same streams. The city refuse in these kampongs is often inadequately collected and flies and mosquitoes are numerous. A high tropical temperature with an average of 27°C, a high relative humidity of about 80% and a high annual rainfall of about 80 inches create ideal conditions for the survival of worm eggs and larvae. Moreover, the unhygienic habits of many people such as eating with the hands, selling food from the ground in the streets and going bare foot encourage the spread of worm infection.

Under such conditions one would expect a high incidence of intestinal worms among the inhabitants of Djakarta and the results obtained in this study are in agreement with this hypothesis. A high incidence is found not only among people of the lower socioeconomic class, who live in the kampongs and have daily contact with contaminated soil (in this study represented by the Indonesians), but also among the middle class (represented by Chinese and European), who live in the better areas of Djakarta. Undoubtedly, the latter groups acquire most of the infections from the kampong areas through various means of transmission such as the consumption of food bought from the kampong people or direct contact with contaminated soil when visiting those areas. The results obtained in this study are comparable to those given by Lie Kian Joe[9] in Djakarta, who, using the same technique, examined 119 Indonesian and 32 Chinese autopsies and found the following results: hookworm: Indonesian 90%, Chinese 69%; *T. trichiura*: Indonesian 84%, Chinese 78%; *A. lumbricoides*: Indonesian 66%, Chinese 50%; *O. vermicularis*: Indonesian 46%, Chinese 41%.

Although the incidence of worm infections is high, the average worm loads for hookworms, *A. lumbricoides* and *T. trichiura* are relatively low. One would expect higher average worm loads since favorable conditions for an effective spread of intestinal worms prevail throughout the kampong areas of Djakarta. This discrepancy is difficult to explain.

Only a small proportion of those infected with hookworms carry a sufficient number of parasites to cause clinical anemia. However, hookworm infection is so widespread in Djakarta (and probably throughout all Indonesia) that this small proportion is in absolute numbers so large as to constitute an important medical problem.

A high hookworm incidence and low average worm load were also found 40 years ago by Darling *et al.*[8] who examined two areas in Djakarta and found an incidence of 100% and 80% in Indonesians and Chinese and an average worm load of 45 and 19.6, respectively. Darling *et al.* also found that the proportion of ancylostomes to necators was higher in Chinese which is comparable to the findings obtained in this study. It is difficult to explain why men show a higher hookworm load than women although the incidence in both sexes is about the same.

An invasion of the intestinal submucosa by *A. duodenale* as described by Bonne and Lie Kian Joe[6] and Lie Kian Joe and Tan Kok Siang[16] was not found in this series of autopsies.

A. ceylanicum and *A. caninum* are not often found in man although they are frequently encountered in cats and dogs. *A. ceylanicum* was previously reported from a man in West Java by Darling *et al.*[8] who found 2 worm specimens. Bonne[2] found a specimen of *A. braziliense* (*A. ceylanicum*?) which had invaded into the intestinal wall. There was one report only on the occurrence of *A. caninum* in man in Indonesia.[14] These hookworm species may occur more frequently than the existing reports would suggest. Infections with these hookworms are easily overlooked and diagnosis is possible only by a careful examination of the ventral teeth present in the oral cavity.

Infections with *A. lumbricoides* and *T. trichiura* are usually due to hand-to-mouth transfer of contaminated soil present in and around human dwellings. Accordingly, the incidence and average worm load are usually higher in children than in adults. In agreement are the results obtained in this study for *A. lumbricoides*, but such a trend is not obvious for *T. trichiura*.

The spread of *O. vermicularis* is a matter of personal hygiene rather than of general sanitation. Accordingly, it is found in all races in about the same frequency.

Strongyloides stercoralis and *Trichostrongylus* species were found from time to time in this survey, but their true incidence could not be determined by the technique employed in this study. *S. stercoralis* infections in Djakarta were studied by different workers. Lie Kian Joe[9]

studied these infections by the stool culture method and by direct examination of scrapings of the duodenal mucosa. He found by the culture method an incidence of 13.1% among 152 Indonesians and 4.8% among 145 Chinese. By direct examination of the duodenum he found 10 cases from 40 autopsies on Indonesian bodies. In 1941 he found 15% in 119 Indonesian autopsies and 13% in 32 Chinese autopsies. Molenkamp[17] employing the stool culture technique found an incidence of 12.6% among 167 Indonesians. Sri Oemijati[19] found by the stool culture technique an incidence of 10.8% among 1659 Indonesians and 8% among 198 Chinese.

Trichostrongylus infections in Djakarta were studied by Lie Kian Joe[10, 11] by direct examination of scrapings of the duodenal mucosa and first part of the jejunum. In 119 Indonesian autopsies, 41% showed *T. colubriformis* and 9% *T. axei*, and in 31 Chinese autopsies 19% showed *T. colubriformis*. The worm load in *Trichostrongylus* infection is usually low.

Oesophagostomum apiostomum was found once in Djakarta.[12]

Intestinal trematodes and cestodes were not found in this survey. They do occur in Djakarta although rarely.

Echinostoma worms are found now and then. It was a mere chance that they were not found in this series of autopsies. In 1941 Lie Kian Joe[9] found three cases in 152 autopsies. Three species have been encountered in Djakarta: *E. lindoense*, *E. recurvatum* and *E. revolutum*.[4] *Haplorchis yokogawai* have been found three times in man.[13] This small parasite may be more frequent than is generally thought. Unusual human trematodes encountered in Djakarta are: *Plagiorchis javensis*,[14, 15, 16] *Phaneropsolus bonnei* and *Paralecithodendrium molenkampi*.[13]

Of the cestodes *Taenia saginata* is most commonly found, in a frequency of two or three cases a year. *Hymenolepis nana* is even more rare. *Raillietina madagascariensis*[3] and *Bertiella studeri*[7] were found once. Two spargana were found in the intestinal wall and when fed to a kitten, yielded 2 specimens of *Diphyllobothrium* subgenus *Spirometra*[5]. Other cestodes have not been found in Djakarta.

Another unusual worm parasite found in an Indonesian man was one belonging to the genus *Acanthocephala*. The species is not known yet. It is different from *Moniliformis dubius* and *Macracanthorhynchus hirudinaceus*, the only two *Acanthocephala* known in man. The worms are being studied and this study will be published later.

Other intestinal trematodes such as *Schistosoma japonicum*, *Paragonimus westermani* and *Clonorchis sinensis* are found from time to time in immigrants from neighboring countries. Autochthonous infections have never been found in Djakarta, although *S. japonicum* is endemic in Central Sulawesi.

SUMMARY

The incidence of intestinal worms was determined in 664 autopsies consisting of 586 Indonesian, 69 Chinese and 9 European, all residents of Djakarta.

Hookworm was found in 89.4% among Indonesians, with an average worm load of 62.7; in 59% among Chinese with an average worm load of 39 and three times in Europeans with an average worm load of 17. The average worm load in men was higher than in women, although the incidence was about the same for both sexes. *Necator americanus* was most often found; *Ancylostoma duodenale*, although frequently found, showed a low worm load; *Ancylostoma ceylanicum* was found seven times and *Ancylostoma caninum* once.

Trichuris trichiura was found in a frequency of 86.9% in Indonesians, with an average worm load of 13.3; 70% in Chinese with an average worm load of seven.

Ascaris lumbricoides was less frequent than *T. trichiura*. The incidence was 63.6% in Indonesians and 42% in Chinese, while the average worm load was six and four, respectively.

Ozyuris vermicularis was also found in a high frequency: 39.4% in Indonesians, 36% in Chinese and 4 cases in Europeans.

Strongyloides stercoralis and *Trichostrongylus* species were also found; however, no trematodes and cestodes were recovered from this series of autopsies.

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BOOK REVIEWS

The Practice of Sanitation; by EDWARD SCOTT HOPKINS AND WILMER HENRY SCHULZE. 3rd edition, 487 pages, illustrated. Baltimore, The Williams & Wilkins Company, 1958. $8.00.

Written as a guide in environmental sanitation for students in sanitary engineering, nurses, sanitarians, and physicians qualifying as health officers, this volume presents in limited detail and 21 chapters, over-all descriptions of the principles, practices and apparatuses used in environmental sanitation in the United States. Among the topics discussed are Principles of Disinfection; sanitation of food, eating places, milk and milk products; Air Pollution; Ventilation; Industrial Sanitation; Insect Control; Rodent Control; and so on. Almost half of the book is devoted to Public Water Supplies, Refuse Disposal, Rural and Urban Sewage Disposal, Stream Pollution, and Industrial Wastes. Discussions are concise and well written and unusually well illustrated. Subject matter is intelligently chosen for readers already having some background in one of the branches of preventive medicine. Each chapter has a list of supplementary reading, generally up-to-date and pertinent.

While experts may find some errors in fields in which the authors are not experts, these defects are generally minor and do not detract materially from the value and usefulness of the book to the reader-group for which it is intended. The volume is well printed and bound in the *sans tache* tradition of the publishers.

MARTIN FROBISHER

Plague Fighter, by WU LIEN-TEH. 667 pages, illustrated. Cambridge, England, W. Hefer & Sons, Ltd., 1959. 30/.

This autobiography covers in great detail nearly 80 years of the author's life. It begins with an account of his participation in the terrible Manchurian pneumonic plague epidemic of 1910. After a description of the International Plague Conference at Mukden in 1911, there is a long flashback to Wu's boyhood in Malaya, his student years at Cambridge and London, and his postgraduate work on the Continent. Following research and later private medical practice in Malaya, Dr. Wu went to China as Vice Director of the Imperial Army Medical School (Tientsin). From this post he was called to Manchuria, where he ultimately became Director of the Plague Prevention Service. In 1930 he was appointed Director of the Chinese National Quarantine Service. After the Japanese occupied Shanghai in 1937, he returned to Malaya and re-entered private practice.

In his wide and frequent travels Dr. Wu has been a keen observer. He seems to have had a prodigious memory or to have made voluminous notes, for he records many details of his experiences, even those of years ago. The book affords vivid glimpses of people and conditions, and gives an insight into the author's alertness. Its characters include Manchu officials, modern Chinese and other political figures, students and scientists from all over the world, and an assortment of personal friends and contacts. We are told what they said, what they wore, what they ate, and how they lived.

The style is unpretentious but clear. It affords easy but not fast reading. Frequent diversions into history and political events, and detailed dissertations on technical and administrative subjects impede the flow of the narrative, and there are a number of repetitions. The book is never dull, though, and often it moves well.

These reminiscences of an authority on plague will interest workers and former workers in this field. Those who have done medical and related work in China will find that the book recalls many memories of people and places. Others may enjoy the glimpses of the world and its events as perceived by a highly educated, cosmopolitan Chinese. But those who find it difficult to relax and read without a compelling purpose, may regard these 639 pages of narrative as formidable.

VICTOR HAAS

Staphylococcus Pyogenes by STEPHEN D. ELEK. 767 pages, illustrated. Edinburgh and London, E. & S. Livingstone Ltd., (Williams & Wilkins Co., Baltimore exclusive U. S. agents), 1959. $15.00.

Dr. Elek has written an impressive and scholarly book on the biology of the staphylococcus and the peculiar host-parasite relationships which appear to surround infections produced by this microorganism. This is a book which will be warmly received by all investigators struggling with the mysteries of staphylococcal disease. For the first time, the diffuse and confused experimental literature on staphylococcal disease has been collected, organized, and critically analyzed.

The chapters on antigenic structure, virulence and disease, and resistance and immune phenomena seem particularly excellent. Many ghosts have been laid to rest in these sections, and Dr.

Elek's broad scientific orientation shows in many of his comments.

In his introduction Dr. Elek modestly claims to dissociate himself from the experimental studies that he reports, but it is pleasing to find that this is not the case. The author's pithy and concise comments on experimental studies are refreshing and valuable, and help to show the reader Dr. Elek's personal orientation. Almost 200 pages of carefully selected references greatly increase the worth of this fine volume.

The criticisms are minor. Dr. Elek occasionally appears arbitrary in his dismissal of certain experimental observations, but his obvious competence in the field entitles him to this liberty, and his opinions are clearly dissociated from the observations which he reports. This reviewer would have been happier if the last chapter on clinical problems in staphylococcal infections had been omitted from what is basically a sophisticated volume on the biology of staphylococcal disease. In this section, occasional comments appear naive. For example, the opinion that the emergence of drug-resistant strains within hospitals could have been avoided by multi-drug therapy of infections would be argued by many investigators. The reviewer would also take issue with Dr. Elek's closing paragraph which reads: "Throughout the course of history, man has gradually reduced his ectoparasites. If the surface carriage of staphylococcus pyogenes is viewed in this light, the essential solution of the problem lies in the abolition of this natural reservoir."

Recent studies on the ecology of human infection suggest to many that human manipulations of disease have forced shifts in the *type* of microbial parasites which can produce serious human disease, but have not actually significantly reduced his ectoparasites. Furthermore, it seems unlikely that a microbe which has been so successful in establishing comfortable symbiotic existence with the human race can be eliminated from man's environment. The reviewer would guess that better understanding of the alterations in host resistance which allow staphylococcal infection to emerge to active staphylococcal disease might be a more profitable approach to control.

But to return to the central thesis, this is a magnificent book. It has been carefully researched, thoughtfully prepared, and delightfully written. It represents a welcome and timely addition to the medical literature and will, I believe, stand as an important contribution for years to come.

DAVID E. ROGERS

Dangerous Marine Animals, by BRUCE W. HALSTEAD, M.D. 146 pages, illustrated. Cambridge, Md., U.S.A., Cornell Maritime Press, 1959. $4.00.

This is a practical manual, every page of which is useful. The technical information it contains is written in simple prose that conveys the meaning clearly to lay and professional reader alike. It is interesting from the preface to the selected bibliography of 23 references.

The four sections comprise: (1) A general chapter on the history of present knowledge of dangerous marine life. (2) A discussion of the Marine Animals That Bite, including the four families of shark most perilous to man, the giant devil ray, barracuda, moray eels, and others. (3) The four major categories of Marine Animals That Sting are described, including the invertebrates: hydroids (as Portuguese Man-O'-War), true jellyfishes (as the sea wasp), univalve shellfish and octopuses, stinging and bristle worms, and sea urchins. The poisonous vertebrates, among which are numbered a large variety of fishes and also the sea snakes, are described here. The geographical distribution and habits of the animals are delineated. (4) The section on Marine Animals That Are Poisonous To Eat provides a considerable amount of condensed information on the marine organisms whose flesh may contain chemical substances, as yet unidentified, which are toxic to man. Included is a brief discussion of the rarer intoxications from consumption of the flesh of sea turtles and certain glandular organs of marine mammals. This chapter is of particular interest since the sea is an important source of food for part of the world's population.

There are many practical instructions for avoiding contact with dangerous sea creatures, and directions for treatment of the victims of such accidents. The mechanisms by which the wounds are inflicted or venomous effects produced are described. The book is extremely well illustrated, and well indexed. Physicians and first aid workers will find it of value for reference; every swimmer, diver and anyone else who spends time in or on the sea should read it.

ELEANOR E. BUCKLEY

NOTICE TO CONTRIBUTORS

The Journal welcomes papers which contribute to the purpose of the American Society of Tropical Medicine and Hygiene. This is the advancement of knowledge of tropical and subtropical medicine and hygiene through investigation, prevention, treatment and control of diseases and deficiencies. It draws upon medicine, biology, public health, nursing, engineering and allied fields of research and application.

The American Journal of Tropical Medicine and Hygiene combines the American Journal of Tropical Medicine and the Journal of the National Malaria Society and therefore has a special concern with the investigation, therapy and control of malaria in all parts of the world, as well as a broad interest in general parasitology and arthropod-borne diseases.

Papers are ordinarily published in the order of reception, which will entail a certain delay in their appearance because of the contractual limitation on the number of journal pages available in any year. Accepted articles which raise no question of scientific priority but for which immediate publication seems important will be inserted in the next available issue of the journal if the author or sponsoring institution will pay the cost, estimate of which may be obtained from the editor. The insertion of such articles does not affect publication of other manuscripts awaiting their turn, since the inserted articles constitute additional pages in the volume without additional cost to the subscriber.

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2. The author should supply, on a separate sheet, a short title not over 50 letters for use as a running head. Also on separate sheets type (a) the authors' names and affiliations; (b) tabular matter; (c) case histories; (d) quotations; (e) formulas and other subsidiary matter in the text; (f) footnotes; (g) references; (h) legends for illustrations; (i) displayed formulas occupying more than 1 line of type, even if using the same size type as the main text.

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